Photosynthetic and fermentative bio-hydrogen production by microalgae and fermenting bacteria

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Photosynthetic and fermentative bio-hydrogen production by microalgae and fermenting bacteria

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Table of Contents

List of Table ---------- **vi**

List if Figure ---------- **viii**

감사의 글 ---------- **xi**

Preface ---------- **xiii**

References ---------- **xviii**

ABSTRACT ---------- **xix**

CHAPTER 1. Introduction ---------- 1

1-1. Issues on Alternative Energy with Biohydrogen Production in the Microbial System ---------- 1

1-2. Specific Objectives ---------- 4

1-3. Overview of Research and Thesis ---------- 6

1-4. References ---------- 8

CHAPTER 2. Literature Review ---------- 11

2-1. Photosynthesis and Hydrogen evolution ---------- 11

2-2. Pathways for H_2 production ---------- 14

2-3. Biophotolysis by Green algae ---------- 15

2-4. O_2 sensitivity of [FeFe]-hydrogenases ---------- 17

2-5. Biochemical and Microbiological Knowledge of the Anaerobic Process ---------- 18

2-5-1. Hydrolysis and Liquefaction ---------- 20

2-5-2. Acidogenesis and Acetogensis ---------- 20

2-6. Process Fundamentals of Anaerobic Treatment ---------- 22

2-6-1. Temperature ---------- 22

2-6-2. pH ---------- 24

2-7. Anaerobic Microbial Consumption of H_2 ---------- 26

2-7-1. Sulfate-Reducing Bacteria ---------- 26

2-7-2. Homoacetogenic Bacteria ---------- 27

2-7-3. Hydrogen Production from Anaerobic Fermentation ---------- 28

2-8. Two-Stage Anaerobic System ---------- 29

2-9. References ---------- 31

CHAPTER 3. Hydrogen Production from Sulfate- and Ferrous-Enriched Wastewater ---------- 37

Abstract ---------- 37

3-1. Introduction ---------- 38

3-2. Materials and Methods ---------- 41

3-2-1. Seed Sludge ---------- 41

3-2-2. Medium Composition and Experimental Batch Setup ---------- 41

3-2-3. Analyses ---------- 42

3-3. Results and Discussion ---------- 47

3-3-1. Effect of SO_4^{2-}, Fe(II), and pH on H_2 production ---------- 47

3-3-2. FISH image analysis of HPB and SRB ---------- 52

3-3-3. Effect of Sulfate Reduction on the Activities of SRB in the H_2 Fermentation Reactor ---------- 56

3-4. Conclusions ---------- 59

3-5. References ---------- 60

CHAPTER 4. Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system ---------- 64

Abstract ---------- 64

4-1. Introduction ---------- 65

4-2. Materials and Methods ---------- 68

4-2-1. Feedstock sampling and preparation ---------- 68

4-2-2. Seed microorganisms ---------- 69

4-2-3. Experimental batch setup ---------- 70

4-2-4. Analytical procedures ---------- 72

4-2-5. Data analysis ---------- 72

4-3. Results and discussion ---------- 74

4-3-1. Characteristics of the different feedstocks ---------- 74

4.3.2. Hydrogen production from different feedstocks ---------- 75

4.3.3. Volatile fatty acids concentrations ---------- 87

4-4. Conclusions ---------- 91

4-5. References ---------- 92

CHAPTER 5. Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions ---------- 98

Abstract ---------- 98

5-1. Introduction ---------- 99

5-2. Materials and Methods ---------- 103

5-2-1. Strain isolation and growth conditions ---------- 103

5-2-2. H_2 and O_2 measurements ---------- 104

5-2-3. Preparation of crude cell extract and measurement of hydrogenase activity ---- 104

5-2-4. Total RNA isolation and hydrogenase mRNA expression ---------- 106

5-3. Results and discussion ---------- 107

5-3-1. Eukaryotic microalgae are capable of hydrogen production under aerobic conditions ---------- 107

5-3-2. Hydrogen production is mediated by hydrogenease at a high oxygen concentration ---------- 113

5-3-3. Specific hydrogenase activity is correlated with initial O_2 levels ---------- 116

5-4. Conclusions ---------- 121

5-5. References ---------- 122

CHAPTER 6. Photo-heterotrophic hydrogen production by a microalga from acetate- and butyrate- enriched wastewater ---------- 125

Abstract ---------- 125

6-1. Introduction ---------- 127

6-2. Materials and Methods ---------- 129

6-2-1. Isolation, purification, and identification of microalga ---------- 129

6-2-2. PCR amplification and phylogenetic analysis ---------- 132

6-2-3. Anaerobic digestion effluent and experimental batch setup ---------- 133

6-2-4. Total RNA isolation and gene expression analysis ---------- 134

6-2-5. Analytical methods ---------- 135

6-3. Results and discussion ---------- 136

6-3-1. Effect of solution pH on the microalgal growth and fatty acid consumption ---- 136

6-3-2. Effect of solution pH on hydrogen production and H_2ase activity ---------- 140

6-3-3. Hydrogen production by microalgae from renewable wastes ---------- 145

6-4. Conclusions ---------- 145

6-5. References ---------- 147

CHAPTER 7. Conclusions ---------- 151

7-1. Hydrogen Production from Sulfate- and Ferrous-Enriched Wastewater ---------- 153

7-2. Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system ---------- 153

7-3. Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions ---------- 154

7-4. Photo-heterotrophic hydrogen production by a microalga from acetate- and butyrate-enriched wastewater ---------- 154

국문요약 ---------- 156

LIST OF TABLES

Table 2-1. Optimal pH for biohydrogen production according the organic substrate --- 25

Table 3-1. Characteristics of the 16S rRNA-directed oligonucleotide probes used for FISH analysis ---------- 46

Table 3-2. Summary of operational performances obtained from wastewater by varying pH, sulfate and iron concentrations ---------- 51

Table 3-3. Quantification of SRB using FISH analysis and batch kinetics analysis ------ 58

Table 4-1. Characteristics of RFW-slurry, corn powder, GFBM and SL ---------- 68

Table 4-2. Summary of operational performances obtained from different feedstock --- 78

Table 4-3. Summary of operational performances obtained from two stage process for Slurry RFW at HRT 18 h ---------- 84

Table 4-4. Organic acid concentrations from stage process for Slurry RFW at HRT 18 h ---------- 89

Table 5-1. The accession number, base pair length of the DNA fragment, and the similarity between amplified sequence and the closest relative sequence of the microalgae species isolated from lake ---------- 102

Table 6-1. The accession number, base pair length of the DNA fragment, and the similarity between amplified sequence and the closest relative sequence of the microalgae isolated from a wastewater treatment plant ---------- 132

Table 6-2. Kinetic parameters for hydrogen production from fatty acids mainly composed with acetate and butyrate at an initial solution pH of 4.9, 6.8, or 8.0 ---------- 142

Table 6-3. Specific activity of H_2ase during the photo-heterotrophic production of H_2 by *M. reisseri* YSW05 ---------- 144

LIST OF FIGURES

Figure 1. Schematic design of energy recovery from organic wastewater by multi-step treatment system ---------- xiii

Figure 2-1. Schematic view of light-powered H_2 production during oxygenic photosynthesis and subsequent formation of carbohydrates in microalgae. The photosynthetic processes are driven by the light energy captured by the light-harvesting complexes of photosystem I (PS I) and photosystem II (PS II) ---------- 13

Figure 2-2. Anaerobic decomposition of organic matter ---------- 19

Figure 3-1. Relationship between (A) H_2 production and (B) SRB concentration at various pHs and sulfate concentrations ---------- 49

Figure 3-2. Fluorescence *in situ* hybridization images of *Clostridium* spp., *Desulfovibrio* sp. and *Desulfobulbus* sp. A) Casc 67 (Cy3, red), EUBmix (Cy3, FITC, green), B) SRB557 (FITC, green), EUBmix (Cy3, red) and C) SRB 660 (6-FAM, green), EUB mix (Cy3, red) at pH 5.8 and sulfate concentration 1000 mg/L, scale bars within each panel indicate distance in microns (μm) ---------- 54

Figure 3-3. Quantitative SRB contents based on total bacteria (Eubmix) from sulfate and ferrous enriched wastewater ---------- 56

Figure 4-1. Schematic diagram of the two-stage fermentation system ---------- 71

Figure 4-2. Accumulative hydrogen productions from different feedstocks at HRTs; (A) 8 h, (B) 18 h, and (C) 24 h ---------- 77

Figure 4-3. Profiles of carbohydrate degradation, H_2 content in two-stage system------- 83

Figure 4-4. Ethanol and VFAs produced from different feedstocks at HRTs; (A) 8 h, (B) 18h, and (C) 28 h ---------- 88

Figure 5-1. Phylogenetic tree showing the relationship among LSU rDNA D1-D2 sequences of isolates YSL01, YSL16 and YSW05, and the most similar sequences retrieved from the NCBI nucleotide database ----------102

Figure 5-2. Net accumulative photoautotrophic hydrogen production by eukaryotic algae cultivated under different initial CO_2 and O_2 concentrations in the headspace --------- 108

Figure 5-3. Variation in the dry cell weight of eukaryotic algae cultivated under different initial CO_2 and O_2 in the headspace (B) *Chlorella vulgaris* YSL01 and (C) *Chlorella vulgaris* YSL16 ---------- 110

Figure 5-4. Photosynthetic hydrogen production and oxygen evolution by two different eukaryotic algae strains and the identification of their hydrogenase mRNA synthesized during oxygenic and photoautotrophic cultivation of the eukaryotes under atmospheric conditions: (a) *Chlorella vulgaris* YSL01 and (b) *Chlorella vulgaris* YSL16 ---------- 115

Figure 5-5. Specific activity of hydrogenase in microalgae as a function of the initial O_2 concentration in the headspace. The parentheses represent the initial CO_2 concentration. Individual data points represent the average of two independent experiments ---------- 117

Figure 5-6. Oxygen sensitivity of in vitro hydrogenase in microalgae cultivated under different initial O_2 in the headspace: (a) *Chlorella vulgaris* YSL01 and (b) *Chlorella vulgaris* YSL16 ---------- 119

Figure 6-1. Cell morphology of *Micractinium reisseri* YSW05 observed under (A) a light microscope (x1500) at pH 8.0, and (B) phylogenetic tree showing the relationship between the LSU rDNA D1-D2 sequence of *Micractinium reisseri* YSW05 and the most similar sequences retrieved from the GenBank database ---------- 131

Figure 6-2. Effect of initial solution pH on the growth rate of *M. reisseri* YSW05: (a) optical density at 680 nm and (b) dry cell weight concentration vs. cultivation time -- 137

Figure 6-3. Biodegradation of (a) butyrate and (b) acetate by *M. reisseri* YSW05 under the aerobic conditions at an initial solution pH of 4.9, 6.8, or 8.0 ---------- 139

Figure 6-4. Cumulative hydrogen production during the photo-heterotrophic growth of *M. reisseri* YSW05 under the aerobic conditions without CO_2 at an initial solution pH of 4.9, 6.8, or 8.0 ---------- 141

감사의 글

교수님께 드리고 싶은 말씀이 아직도 끝이 없는 가운데 감사의 표현도 드리게 되어 너무나 죄송한 마음입니다. 제가 처음 박사과정을 시작 하던 때에 제 의견을 충분히 반영하여 좋은 연구를 진행할 수 있도록 환경을 만들어 주시고 지도해주시던 열정 마음 속 깊이 간직하겠습니다. 연구와 논문 작업 중간중간 뜻대로 진행되지 않는 저에게 교수님의 학창시절 이야기와 해결방법을 말씀해주시며 용기를 주시고 아무것도 모르던 저에게 문제 하나하나를 전해 주시며 연구과정에서의 답을 찾는 법을 알게 해주시던 그 모습, 발표에 긴장을 많이 하였던 저를 묵묵히 격려해 주시면서 때로는 따끔한 충고로 긴장의 끈을 놓지 않고 연구에 대한 열정을 잃지 않게 다독여 주시던 그 모습 간직하겠습니다.

교수님께서 항상 말씀하시던 교육철학과 가르침을 소중히 간직하여 교수님의 은혜가 헛되이 되지 않는 제자가 반드시 되겠습니다.

대학원에 입학했을 때가 엊그제 같은데 벌써 시간이 흘러 졸업을 하게 되었습니다. 부족한 제가 논문을 쓰도록 해주신 모든 분들께 이 자리를 빌어 감사의 마음을 전하려 합니다.

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정아에게도 너무나 고맙고 감사합니다. 항상 힘이 되는 말로 말로 저를 끝까지 격려해 주신 아버님과 어머님의 지혜와 사랑으로 학위과정을 하게 되어 너무 감사합니다.

여러 가지 실험 방법과 과제 수행 능력을 키워주시고 논문 작업에 많은 도움을 주신 연구실 동료들에게 감사드립니다. 대학원 생활에 있어서 소중한 추억을 간직하게 해주고 나의 옆에서 항상 지원군이 되어준 정우식, 지민규, 이상훈, 조동완, 안용태, 김성욱, 김용림과 멀리있어 자주 보지 못해 항상 아쉬웠던 윤현식, 지은도, 이우람, 그리고 이제 공부를 시작하는 권오훈, 이민선, 열혈공부중인 외국인학생 Abinashi, Sayed 에게 고마움을 전하며 좋은 연구하길 바랍니다. 특히 저와 많은 추억을 쌓은 상훈이가 옆에 있어 많은 도움이 되었습니다.

마지막으로 주위의 모든 분들께 감사드립니다. 하나의 열매를 맺었듯이 더욱 열심히 하겠습니다.

Preface

This Ph.D. thesis is the result of a research project carried out at the Department of Environmental Engineering, Yonsei University (YU), during the period from March 2009 to June 2013. Professor Byong-Hun Jeon was the main supervisor.

This study over goal is developing the novel technologies for the best energy recovery using organic wastewater. The present study provides a fundamental scientific blueprint for the development of an integrated set of technologies designed to recover energy from organic wastewater resources (Figure 1).

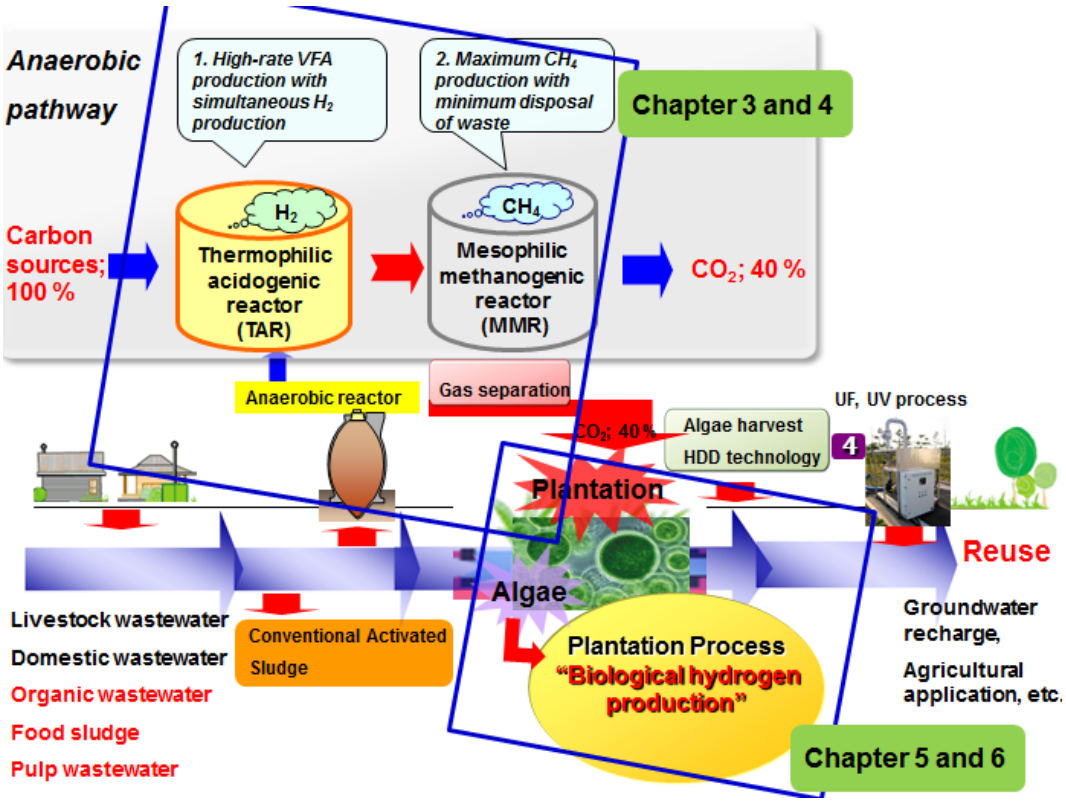

Figure 1. Schematic design of energy recovery from organic wastewater by multi-step treatment system (Jeon et al., 2010).

Wastewater generation from food factory, livestock and wastewater treatment plants increases every year, and its disposal has been a central issue in Korea for many years. Ocean dumping, landfill, and incineration have been the most widely used methods, but the needs for new and environmentally friendly treatment methods are growing rapidly due to the pollutions of soil, groundwater, stream, river, ocean and air. As an alternative method, researches on the production and utilization of hydrogen and methane from organic matter in wastewater have been intensively carried out considering the wastewater as an alternative energy source (Lin and Chen, 2006).

Many studies have shown the use of organic wastewater for bioenergy (hydrogen and methane) production (Mizuno and Noike, 1998; Hawkes et al., 2002). Biological hydrogen production utilizes organic wastewater or other wastes as raw materials which contain a variety of organic substrates (Lin and Chen, 2006). The high sulfate content in wastes produced from pulp/paper, sea-food processing and alcohol fermentation industries (Chen et al., 2008) has been found to adversely affect the anaerobic digestion (Bitton, 1994). Treatment of sulfate containing wastewater by anaerobic fermentation results in SRB proliferation. The decreased H_2 production at pH 6, as compared with pH 5.5, was attributed to the increase of SRB activity, which competed against the activity of H_2-producing bacteria (HPB). In order to obtain an appropriate fermentation system from sulfate and ferrous-enriched wastewater, it would be beneficial to quantitatively predict SRB activity during the H_2 fermentative process for optimizing substrate

utilization in the H_2 reactor. Food waste also has proven to be highly desirable substrate for anaerobic fermentation due to its high digestibility and well balanced carbon and nutrient contents (Zhang et al., 2007). The efficiency of the fermentation process depends on the food to microorganism ratio and hydrogen production is highly variable depending on this ratio (Pan et al., 2008). To our knowledge the operation of a two-stage (dark/dark) fermentation system for H_2 production from carbohydrate-rich feedstocks has not been reported. In order to select and design an appropriate two-stage (dark/dark) fermentation system, it would be beneficial to predict H_2 production during the two-stage fermentative process not only for the purpose of substrate reutilization in the second stage but also for optimizing the combined system.

This research aim is to produce the biohydrogen using photobiological hydrogen technology from various wastewaters (i.e., high concentration of sulfate and food wastewater) via anaerobic fermentation and microalgae process. However, anaerobic fermentation process involves the generation of CO_2, a greenhouse gas, which is the drawback of this technology (Hawkes et al., 2002). Reduction of carbon dioxide emission is therefore of international concern, and the removal or reuse of carbon dioxide is demanded. Our another research aim is to use CO_2 generated from the anaerobic bio-energy generating process as a carbon source for the algae (Rittmann, 2008) and algal growths with other nutrient (short chain fatty acids) sources present in the effluent form fermentation process. In order to evaluate the optimal the potential photo-autotrophic hydrogen

production during algae cultivation under aerobic system for algae plantation and photo-heterotrophic hydrogen production from short chain fatty acid (acetate- and butyrate-) enriched wastewater using microalgae, it would be beneficial to have a good understanding on microalgal activity during the hydrogen production for optimizing substrate utilization.

In conclusion, we are aiming to achieve both bio-energy production and carbon dioxide emission control with simultaneous wastewater treatment. This study envisions a bold, new leap forward by coupling the novel technologies with the existing engineering systems for multiple energy recovery.

The thesis is organized in two parts. The first part is a dissertation providing background for understanding the important aspects of the biohydrogen process by fermentative bacteria and the photosynthetic hydrogen process. The second part consists of the following papers.

Part 3: Jae-Hoon Hwang, Jeong-A Choi, You-Kwan Oh, Reda A.I. Abou-Shanab, Hocheol Song, Booki Min, Yunchul Cho, Jeong-Geol Na, Jakon Koo, Byong-Hun Jeon. Hydrogen production from sulfate- and ferrous-enriched wastewater. Int. J Hydrogen Energy 36 (2011) I3984-I3990.

Part 4: Jae-Hoon Hwang, Jeong-A Choi, Reda A.I. Abou-Shanab, Booki Min, Hocheol Song, Yongje Kim, Eung Seok Lee, Byong-Hun Jeon. Feasibility of hydrogen production from ripened fruits by a combined two-stage

(dark/dark) fermentation system. Bioresour.Technol. 102 (2011) 1051-1058.

Part 5: Jae-Hoon Hwang, Hyun-Chul Kim, Jeong-A Choi, R.A.I. Abou-Shanab, Brian A. Dempsey, John M. Regan, Jung Rae Kim, Hocheol Song, In-Hyun Nam, Su-Nam Kim, Woojung Lee, Donghee Park, Yongje Kim, Jaeyoung Choi, Min-Kyu Ji, Woosik Jung, Byong-Hun Jeon. Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions. “Nature communications” Submitted.

Part 6: Jae-Hoon Hwang, Jeong-A Choi, Hyun-Chul Kim, Yong-Rim Kim, Byong-Hun Jeon. Photo-heterotrophic hydrogen production by a microalga from acetate- and butyrate- enriched wastewater. going to be submitted to “Biotechnology for Biofuels”.

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Jeon, B.H., Min, B., Song, H., Hwang, J.H., Kim, Y.H., Abou-Shanab, R.A.I., Multiple energy recovery and carbon control (MERCC) in wastewater treatment process, Korea Patent Registration Number (2010) 10-1102310.

Lin, C.Y., Chen, H.P., Sulfate effect on fermentative hydrogen production using anaerobic mixed microflora. Int. J. Hydrogen Energy, 31 (2006) 953-960.

Mizuno, O., Li, Y.Y., Noike, T., The behavior of sulfate-reducing bacteria in acidogenic phase of anaerobic digestion. Water Res. 32 (1998) 1626-1634.

Pan, J., Zhang, R., El-Mashad, H.M., Sun, H., Ying, Y., Effect of food to microorganism ratio on biohydrogen production from food waste via anaerobic fermentation. Int. J. Hydrogen Energy, 33 (2008) 6968-6975.

Rittmann, B.E., Opportunities for renewable bioenergy using microorganisms. Biotech. Bioeng. 100 (2008) 203-212.

Zhang, R., El-Mashad, H.M., Hartman, K., Wang, F., Liu, G., Choate, C., Gamble, P., Characterization of food waste as feedstock for anaerobic digestion. Bioresour. Technol. 98 (2007) 929-935.

ABSTRACT

Photosynthetic and fermentative bio-hydrogen production by microalgae and fermenting bacteria

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A good understanding of both, photosynthetic and fermentative bio-hydrogen metabolism is essential in various research areas such as microbial metabolism, auto-/hetero- photosynthesis, technologies of both alternative energy production and conventional organic waste treatment, and biochemistry. Biological hydrogen production processes can be classified such as biophotolysis of water using algae and cyanobacteria, photodecomposition of organic compounds by photo-synthetic bacteria, fermentative hydrogen production from organic compounds, and hybrid systems using photosynthetic and fermentative bacteria. The demand for more efficient hydrogen production by dark hydrogen fermentation is particularly increasing. However, full scale bio-hydrogen plants fail to exist due to economical issues. A two-stage process to enhance the bio-hydrogen production is one possible solution for increasing the efficiency of dark fermentation process. However, anaerobic fermentation process involves the generation of CO_2, a greenhouse gas, which is the drawback of this technology. Reduction of carbon dioxide

emission is therefore of international concern, and the removal or reuse of carbon dioxide is demanded. Our another research aim is to use CO_2 generated from the anaerobic bio-energy generating process as a carbon source for the algae and algal growths with other nutrient (short chain fatty acids) sources present in the effluent form fermentation process. Mostly, capability of hydrogen production by green algae has been demonstrated focusing on photo-heterotrophic and -mixotrophic production under anaerobic and limited aerobic conditions. In order to evaluate the optimal the potential directly photo-autotrophic hydrogen production during algae cultivation under aerobic system for algae plantation and photo-heterotrophic hydrogen production from short chain fatty acid (acetate- and butyrate-) enriched wastewater using microalgae, it would be beneficial to have a good understanding on microalgal activity during the hydrogen production for optimizing substrate utilization.

This study over goal is developing the novel technologies for the best energy recovery using organic wastewater. The present study provides a fundamental scientific blueprint for the development of an integrated set of technologies designed to recover energy from organic wastewater resources. We were investigated optimal condition on fermentation process for bioenergy using various organic wastewater (i.e., sulfate wastewater and fruit waste) and direly hydrogen generation from microalgae using inorganic and organic carbon sources. This study showed the effects of varying sulfate concentrations with pH on continuous fermentative hydrogen production were studied to understand the relationship between sulfate reducing bacteria (SRB) and hydrogen (H_2) production. Both Fe(II) and SO_4^{2-} improved the H_2 production efficiency from wastewater. In order to obtain an appropriate fermentation system using sulfate- and ferrous- enriched wastewater, it would be beneficial to quantitatively predict SRB activity during the H_2 fermentative process for optimizing substrate utilization in the H_2 reactor. The inhibition

in hydrogen production by SRB at pH 6.2 diminished entirely by lowering the pH to 5.5, at which activity of SRB is substantially suppressed.

Enhancement of fermentative bioenergy production is essential for efficient hydrogen production. A two-stage fermentation system was employed for combined hydrogen production to improve the energy efficiency. The energy efficiency (H_2 conversion) obtained from mixed ripened fruits (RF) as substrate increased from 4.6% (in thefirst stage) to 15.5% (in the second stage).

A direct hydrogen study showed that microalgal strains can upregulate the expression of mRNA synthesis of hydrogenase gene (*hydA*) and simultaneously produce hydrogen through photosynthesis using CO_2 as the sole source of carbon with continuous illumination under aerobic conditions. We employed dissolved oxygen regimes typical of environmental conditions for microalgae in natural aquatic systems. The expression of *hydA* and the specific activity of hydrogenase evidenced that microalgae enzymatically produced hydrogen even under atmospheric conditions, which has been previously considered infeasible.

Microalgal photo-heterotrophic hydrogen production by green algae organic carbon enriched (i.e., acetate and butyrate) wastewater effluent under anaerobic conditions was investigated. The wastewater was abundant in acetate and butyrate which caused different state of algae growth for phototrophic H_2 production. The accumulative H_2 production by microalgae with high organic concentration was almost 1.8 times higher than that from the control. These results demonstrate that AWE could be an effective substrate for enhancing the photo-heterotrophic H_2 production from green algae.

Key words: Hydrogen; Photosynthesis; Fermentation; Microalgae; Fermenting bacteria; Autotrophic; Hydrogenase; mRNA expression; Sulfate reducing bacteria; Two stage

CHAPTER 1

Introduction

1-1. Issues on Alternative Energy with Biohydrogen Production in the Microbial System

Hydrogen is a clean and sustainable energy source for various industrial activities with very high energy capacity per unit mass (118.2 kJ/g) (Park et al., 2005). It is a non-polluting fuel and can be used in fuel cells for the production of electricity (Layet al., 1999). Conventional and present sources of hydrogen production (e.g., water electrolysis or chemical cracking of hydrocarbons) require electricity derived from fossil fuels or nuclear fission; thus biohydrogen production is gaining wide attention due to recent concerns over global warming (Dincer, 2002; Hawkes et al., 2002). Hydrogen can be produced biologically through microbes either by photosynthetic bacteria cultured under anaerobic conditions or by anaerobic fermentative bacteria.

Photo biological production of H_2 by eukaryotic algae is of interest because it holds the promise of generating a renewable fuel from abundantly available light and water (Kosourov et al., 2003; Hallenbeck, 2004). An alternative approach of photo producing H_2 is based on the concept of indirect bio photolysis, in which metabolite accumulation acts as an intermediate step between photosynthetic H_2O oxidation and H_2 production (Melis et al., 2000). Unicellular microalgae hold the

attention for commercial production of hydrogen and biomass (Oncel and Sukan, 2009). Photosynthetic H_2 production by microalgae is a promising process due to its minimal nutritional requirements. Electrons released upon the oxidation of water are transported to Fe-S protein ferredoxin on the reducing side of photo system I (Ghirardi et al., 2002). The O_2 evolution on the oxidizing side of photo system II and H_2 production on the reducing side of PS I with the ratio of H_2:O_2 = 2:1 has not yet been achieved. This is because of the O_2 sensitivity of hydrogenase (Melis et al., 2000).

In micro algal production system, the achievable photosynthetic productivity and light utilization efficiency of the algae are the most important factors in cost determination (Lindblad, 2004; Polle et al., 2002). The algae also have the ability to operate in two distinct environments, namely aerobic and anaerobic (Melis, 2007). Alteration of the photosynthesis respiration relationship lead to a continuous H_2 photo production process that sustained for many days (Ghirardi et al., 2000). The processes of oxygenic photosynthesis, mitochondrial respirations, catabolism of endogenous substrates, and electron transport via, Fe-hydrogenase pathway lead to H_2 production (Melis and Happe, 2001). The evolution of H_2 in light/dark cycle was the early method for hydrogen production by green algae (Polle et al., 2002). It was based on the fact that algal cells accumulate starch during their growth in light. The expression of the Fe-hydrogenase is elicited in the light, leading to H_2 production by the algae (Melis et al., 2000).

In contrast to photolytic production of H_2, anaerobic fermentative processes have fast production rates, reduced waste generation and no requirement of additional light energy (Das and Verziroglu, 2001).Fermentative hydrogen production from organic substances results in the incomplete decomposition of substrate into organic acids such as acetate and butyrate. Butyrate is more dominant because of its lower Gibbs free energy (ΔG= −257.1 kJ) compared to acetate (ΔG= −184.2 kJ) and its production involves enzyme activity (Nandi and Sengupta, 1998).

Biological hydrogen production utilizes organic wastewater or other wastes as raw materials which contain a variety of organic substrates (Lin and Chen, 2006). The high sulfate content in wastes produced from pulp/paper industries, sea-food processing and alcohol fermentation industry have been found to adversely affect the anaerobic digestion (Chen et al., 2008). Treatment of sulfate containing wastewater by anaerobic fermentation results in SRB proliferation. In previous reports, most of the acidogenic procedures showed decreased hydrogen and methane gas production in sulfate rich wastewater at pH 6-7 (Esposito et al., 2003). Mizuno et al. (1998) investigated the effects of COD/SO_4^{2-} ratio and HRT in acidogenic phase and clearly suggested that sulfate reducing bacteria can adversely influence on the pathway of sucrose degradation leading to lower hydrogen production.

1-2. Specific Objectives

Several researches reported that the utilization of waste materials containing high concentrations of organics, such as municipal solid waste, industrial wastewater, and agricultural waste, to produce hydrogen may warrant significant economic and environmental benefits (Ting and Lee, 2007; Hwang et al., 2009). Acid waste stream of a synthetic drug plant and yellow ginger processing wastewater contain high sulfate concentration (Zhao et al., 2008). Only limited information is available for the effect of high sulfate concentrations on microbial hydrogen production. Food waste has proven to be highly desirable substrate for anaerobic fermentation due to its high digestibility and well balanced carbon and nutrient contents (Zhang et al., 2007). The efficiency of the fermentation process depends on the food to microorganism ratio and hydrogen production is highly variable depending on this ratio (Pan et al., 2008). To our knowledge the effect of variation of solution pH under very high sulfate concentrations and the operation of a two stage (dark/dark) fermentation system for H_2 production from different ripened fruits have not been reported.

The direct photo-auto/heterotrophic hydrogen production from microalgae would be beneficial to understand the microalgal activity during the hydrogen production for optimization of substrate utilization, enzyme activity, hydrogen production rate, and hydrogen yield.

This study can help to understand the biohydrogen production from two mechanisms (i.e., photosynthesis and fermentation) in the microbial system,

further Also, this research will be beneficial to understand direct hydrogen production from microalgae between auto- and hetero- trophic condition, and improvement of hydrogen using enriched sulfate waste and ideal two-stage (H_2/H_2) process via fermentation. The understanding biologic hydrogen production process is important to develop an industry of the alternative energy. The specific objectives of this research were to:

[Chapter 3-4 : Fermentative hydrogen production]

1) To investigate the effect of SRB activity at various concentrations of sulfate and ferrous on fermentative hydrogen production under controlled pH.
2) To study a sustainable two-stage fermentative hydrogen production process reusing the sludge.

[Chapter 5-6 : Photosynthetic hydrogen production]

1) To evaluate production of hydrogen under the aerobic, photo autotrophic conditions.
2) To investigate the efficiency of butyrate and acetate of mixture fatty acids obtained from anaerobic digester.
3) To study the relative H_2 production between H_2ase activity and microalga growth at different pH values.

1-3. Overview of Research and Thesis

This thesis contains 7 Chapters. The literature review is described in Chapter 2 including the description of photosynthesis pathways for hydrogen production and anaerobic process. The primary objective of Chapter 3 and Chapter 4 were to investigate influence of sulfate concentration on fermenting bacteria and enhancement of energy recovery using two-stage (dark-/dark fermentation) bioreactor on hydrogen production. Since we observed complete inactivation of sulfate reducing bacteria under low pH (<5.5) in the fermentation process, H_2 production was not influenced by the low specific sulfate reducing activities for the study that appeared in Chapter 3, the possibility of hydrogen production was irrespective of a low SRB activity. The preliminary result of two-stage hydrogen production demonstrated that the hydrogen production from the first stage effluent amended with the digested sewage sludge and showed a promising H_2 production that is the main contents of Chapter 4.

Experimental results on biohydrogen production onto microalgae are discussed in Chapter 5 and 6. A direct hydrogen production from microalgae, in Chapter 5, provides an evidence of naturally evolved oxygen-tolerant hydrogenase in eukaryotic microalgae, and further study may open a new avenue of continuous biophotolysis for hydrogen production and an opportunity to apply artificially designed oxygen tolerant hydrogenase-based biomimetic photovoltaic cell using eukaryotic algae. The research results showed that the organic materials (i.e., fatty acids) from fermentation effluent can be used as a valuable feedstock for photo-

heterotrophic cultivation of microalgae and subsequent bioenergy production that is main subjects of Chapter 6.

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CHAPTER 2

Literature Review

2-1. Photosynthesis and Hydrogen evolution

Photosynthesis, either oxygenic or anoxygenic, can be generalized as the conversion of light into chemical energy through a series of reduction–oxidation (redox) reactions. Light is first absorbed by pigment molecules associated with the photosystem protein complex, leading to the formation of electrons (out of electron donors) that are shuttled through an electron transport chain for generating ATP and NAD(P)H. These energy-rich intermediate compounds are then consumed to drive a selection of reductions. The most important one is known as carbon fixation through which CO_2 is reduced to synthesize glucose and its polymeric forms. Other examples include the reduction of nitrite ions or molecular nitrogen to form ammonia (known as nitrogen fixation) and the reduction of sulfite ions to form hydrogen sulfide (Lawlor, 1993). Oxygenic photosynthesis, carried out by eukaryotic plants, algae, and cyanobacteria, involves the use of water as an electron donor, leading to the generation of molecular oxygen. Some ancient strains of cyanobacteria, however, can shift between oxygenic photosynthesis and anoxygenic bacterial-type photosynthesis (e.g. using hydrogen sulfide as an electron donor), suggesting an evolutionary

continuum between aerobic phototrophic plants and algae and anaerobic phototrophic bacteria (Lawlor, 1993).

The PS II RC contains several redox components, including a special form of Chl a, P680 (the primary electron donor of PS II), pheophytin (Pheo) a as well as the primary and secondary plastoquinone electron acceptors (QA and QB, respectively) (Kruse et al., 2005; McEvoy and Brudvig, 2006). The electron from excited P680 in PS II RC is transferred through a number of carriers to the cytochrome complex, which consists of several subunits including cytochrome f and cytochrome b6 (Cytb6/f). Electrons derived from water are transferred to the oxidized P680. Finally, a series of redox reactions within the cytochrome complex ultimately transfer the electrons to a plastocyanin (PC) that acts as a shuttle to PS I.

In PS I, electrons are transferred from PS I RC to ferredoxin (Fd), a protein that carries electrons to other reaction pathways outside the thylakoid. The reaction center replaces the electrons transferred to Fd by accepting electrons from the PC located between the cytochrome complex and PS I. Normally, Fd shuttle's electrons to the enzyme ferredoxin-$NADP^+$-oxidoreductase that reduces $NADP^+$ to NADPH, an important source of reduction needed to convert CO_2 to carbohydrates in the Calvin-Benson cycle. As electrons are transported through the electron-transport chain, protons (H^+) outside the thylakoid are carried to the inner thylakoid space forming a proton gradient across the thylakoid membrane (Fig. 2-1).

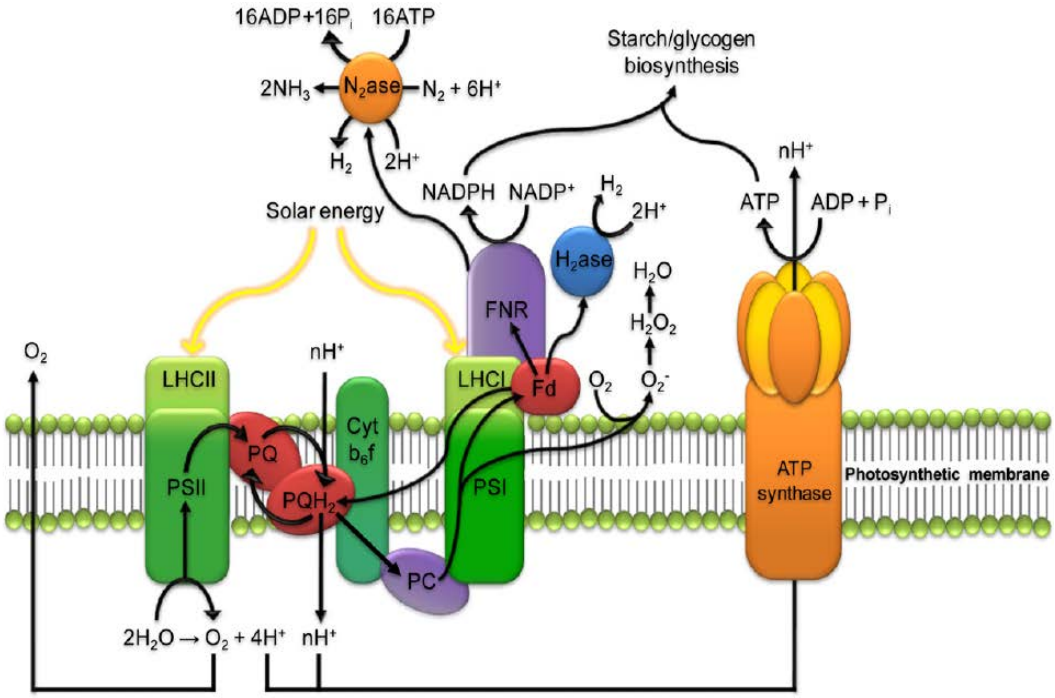

Figure 2-1. Schematic view of light-powered H_2 production during oxygenic photosynthesis and subsequent formation of carbohydrates in microalgae. The photosynthetic processes are driven by the light energy captured by the light-harvesting complexes of photosystem I (PS I) and photosystem II (PS II) (Srirangan et al., 2011).

2-2. Pathways for H_2 production

Several hydrogenase-dependent pathways were analyzed in view of improving H_2 production in cyanobacteria and algae (Kruse et al., 2005; Rupprecht et al., 2006). The first pathway is the photo-dependent H_2 production in which the electron transport occurs via the two photosystems from water to Fd. H^+ that is released from the thylakoid lumen and e^- from reduced Fd are used for H_2 production by the hydrogenase. This is efficient pathway in green algae under conditions of low PS II activity, for instance, upon sulfur deprivation, which significantly eliminates O_2, improving the rate of H_2 production (Melis et al., 2000; Ghirardi et al., 2007).

The second pathway uses the nitrogenase of cyanobacteria (Melis and Happe, 2001; Allakhverdiev et al., 2009) in which the electrons and protons are delivered from photosynthesis. However, this pathway requires large number of photons which results in lower efficiency compared to other pathways and hence, makes it economically impractical.

The third pathway for H_2 production is photo-fermentative, which effectively occurs in two temporal stages. During the first stage, the photosynthetic processes produce carbohydrates for mitochondrial respiration and cell growth. During the second stage, mitochondrial oxidative phosphorylation is largely inhibited and under anaerobic conditions hydrogenase expression is induced. PS I accepts e^- and H^+ delivered to the PQ pool, which is fully reduced under anaerobic conditions by enzymatic oxidation of intracellular reductants derived from fermentation, and

reduces the hydrogenase through Fd. Temporal separation of H_2 and O_2 flows is crucial for increasing the efficiency of this pathway.

2-3. Biophotolysis by Green algae

Green algae are capable of photo-producing appreciable quantities of hydrogengas under certain conditions (Gaffron, 1940). It was determined that light-dependent hydrogen production resulted only when cells were pre-incubated anaerobically in the absence of light, a requirement for induction of [FeFe]-hydrogenase gene expression. Two distinct hydrogenase-encoding genes (i.e., *hydA1* and *hydA2*) have been located within the genome of the green alga. Although the hydrogenase enzyme is localized in the chloroplast stroma, both *hydA1* and *hydA2* are encoded within the cell's nucleus, as is true of over 90% of all chloroplast proteins which are translated in the cytoplasm and trafficked posttranslationally into the chloroplast via a signal peptide (Happe et al., 1994, Smith, 2006).

Biophotolysis, the biological splitting of water to yield hydrogen and oxygen gas, refers to the coupling of photosynthesis and hydrogen evolution. However, oxygenic photosynthesis is intrinsically antagonistic to photo-hydrogen production due to the concomitant production of molecular oxygen upon oxidation of water. The evolved oxygen potently and irreversibly inhibits hydrogenase activity. The extreme oxygen sensitivity exhibited by most [FeFe]-hydrogenases is often cited as the single greatest challenge impeding the feasibility of an industrial

biophotolysis process for the production of biohydrogen. Other challenges, predominantly low photochemical efficiencies, have been shown to hinder the feasibility of direct biophotolysis using green algae. For biophotolysis to compete with current petrochemical methods as a sustainable platform for the production of biohydrogen, such challenges must be resolved through a combination of genetic and bioprocess engineering methodologies. This section discusses current limitations of hydrogen production via biophotolysis and reviews corresponding strategies to improve photo biological hydrogen production from green algae.

2-4. O_2 sensitivity of [FeFe]-hydrogenases

Oxygen sensitivity of [FeFe]-hydrogenases is a multifaceted challenge, as hydrogenase transcription, maturation, and photocatalytic activity are all highly susceptible to inhibition by molecular oxygen. Relative to the cyanobacterial [NiFe]-hydrogenases, [FeFe]-hydrogenases have been shown to be much more sensitive to oxygen, as significant activity is lost within only a few seconds exposure to oxygen (Cohen et al., 2005). Oxygen is speculated to bind to an iron atom within the double Fe subcluster and in turn competitively inhibit proton binding required for generation of H_2. Furthermore, oxygen has been shown to be involved in hydrogenase gene expression, as *HydA* and *HydEFG* gene transcription is triggered, at least in part, by anoxia. For this reason, anoxic conditions are desirable in order to trigger hydrogenase gene expression and prevent enzyme inhibition. However, such conditions are economically unfeasible, as sparging with an inert gas such as helium is costly for a large scale production process. Two distinct means of overcoming this inherent challenge have been successfully applied in recent years, namely sulfur deprivation and hydrogenase protein engineering.

2-5. Biochemical and Microbiological Knowledge of the Anaerobic Process

The anaerobic process is degradation of organic substrates in the absence of oxygen to carbon dioxide and methane with only a small amount of bacterial growth (Gray, 2004). The digestion process consists of several interdependent, complex, sequential and parallel biological reactions. During these reactions the products from one group of microorganisms serve as the substrates for the next (Noykova et al., 2002). The overall conversion process is often described as a three stage process which occurs simultaneously within the anaerobic digester (Mtui, 2009). The first is the hydrolysis of insoluble biodegradable organic matter, the second is the production of acid from smaller soluble organic molecules, and the third is methane generation. The three stage scheme involving various microbial species can be described as follows (see Fig. 2-1): (1) hydrolysis and liquefaction; (2) acidogenesis, and (3) methane fermentation.

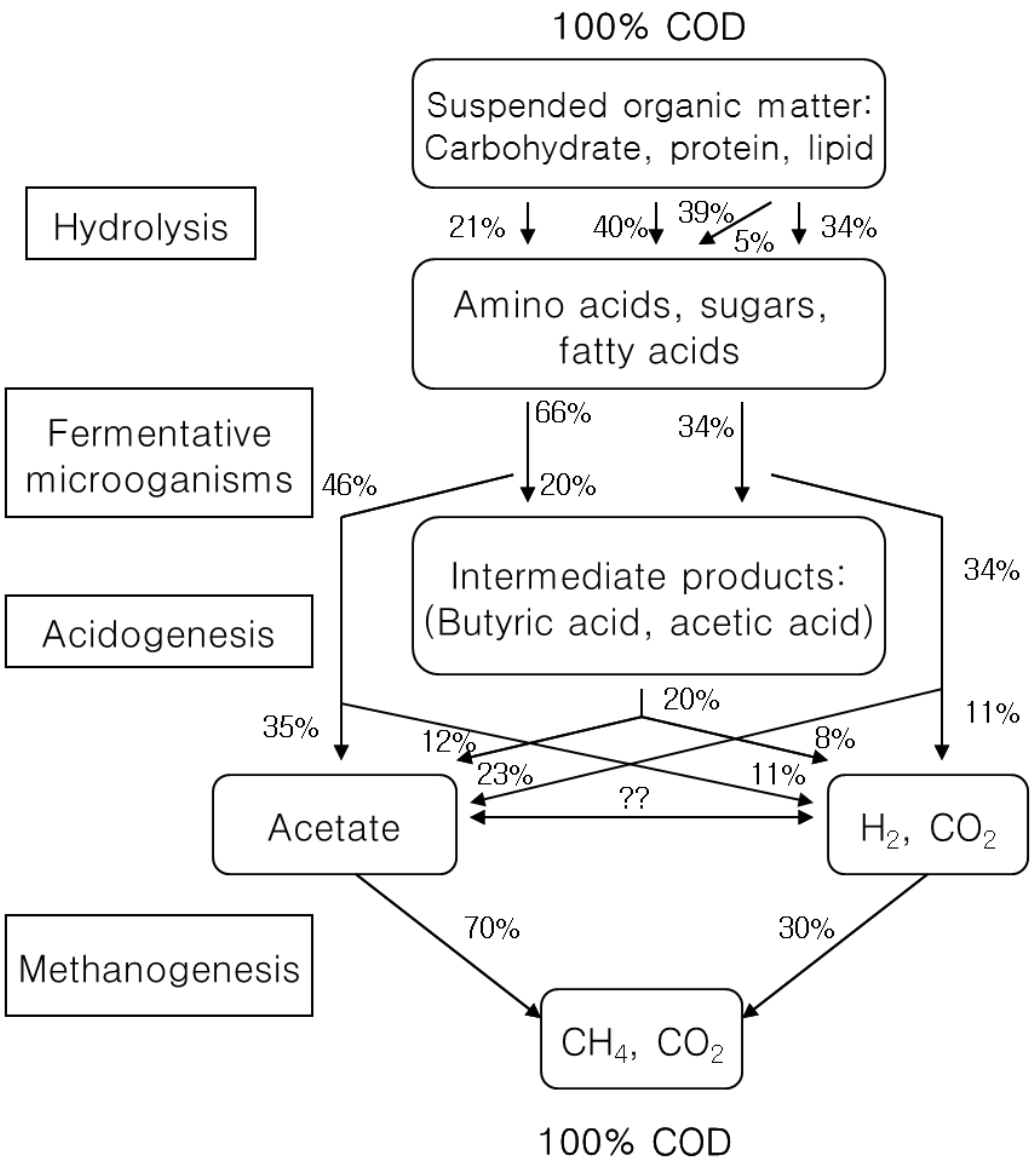

Figure 2-2. Anaerobic decomposition of organic matter (Zehnder et al. 1982).

2-5-1. Hydrolysis and Liquefaction

Hydrolysis and liquefaction are the breakdown of large, complex and insoluble organics into small molecules that can be transported into the microbial cells and metabolized (Droste, 1997). Bacteria transform the particulate organic substrate into liquefied monomers and polymers (i.e. proteins, carbohydrates) are transformed to amino acids, monosaccharides and fatty acids during hydrolysis. Some of the enzymes involved are cellulase, amylase, protease, and lipase (Mtui, 2009). Essentially, organic waste stabilization does not occur during hydrolysis, and the organic matter is simply converted into a soluble form that can be utilized by the bacteria (McCarty and Smith, 1986; Parkin and Owen, 1986).

2-5-2. Acidogenesis and Acetogensis

Acidogenic bacteria transform the products of the first reaction (hydrolysis) into short chain volatile acids, ketones, alcohols, hydrogen and carbon dioxide. Hydrogen is produced by the acidogenic and hydrogen-producing acetogenic bacteria (Hwang et al., 2009). The principal products of acidogenesis stage are propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), acetic acid (CH_3COOH), formic acid (HCOOH), lactic acid ($C_3H_6O_3$), ethanol (C_2H_5OH) and methanol (CH_3OH), among others (Bonmati et al., 2001). From these products, the hydrogen, carbon dioxide and acetic acid will skip the acetogenesis stage, and be utilized directly by the methanogenic bacteria in the final stage. This mechanism is commonly referred to as inter-species hydrogen transfer.

Acetogenic microorganisms can also tolerate a wide range of environmental conditions (Novaes, 1986; Parkin and Owen, 1986). The main pathway of acetogenesis, volatile fatty acids is converted into acetate, carbon dioxide, and hydrogen (Hwang et al., 2009). The overall performance of the anaerobic digestion system is affected by the concentration and proportion of individual volatile fatty acids formed in the acidogenic stage because acetic and butyric acids are the preferred precursors for methane production (Hwang et al., 2001). A theoretical maximum of 4 moles of hydrogen is obtained from acetic acid and 2 moles of hydrogen from butyric acid due to the butyrate pathway (Eqs. (1) and (2) below) (Hawkes et al., 2007).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

The accumulation of lactate, ethanol, propionate, butyrate, and higher volatile fatty acids is the response of the bacteria to increased hydrogen concentration in the medium (Schink, 1997). Propionate is a metabolite of a hydrogen-consuming pathway, while ethanol and lactic acid are involved in a zero-hydrogen balance pathway (Eqs. (3) - (5)).

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \quad (4)$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2 \quad (5)$$

2-6. Process Fundamentals of Anaerobic Treatment

There are many environmental and operational variables associated with anaerobic treatment. The important factors currently known include temperature, pH, alkalinity, and nutrient requirements (Demirel and Yenigun, 2002).

2-6-1. Temperature

Temperature is often considered as one of the most important parameters affecting both biohydrogen production yields and microbial metabolisms in mixed cultures (Li and Fang, 2007). A uniform temperature is essential for consistent and efficient reactor operation. Most studies on fermentative hydrogen production have been based on mesophilic temperatures.

Microorganisms used in this degradation are divided into several categories depending on their optimal temperature. Psychrophilic organisms grow best in temperatures (0-20 °C), mesophilic (20-42 °C), and thermophilic (42-75 °C) (Hulshoff-Pol, 1998). Anaerobic reactors most often operate at mesophilic and thermophilic ranges (van Lier et al., 1996). Methanogenesis is possible under

psychrophilic conditions but occurs at lower rates. Bacterial activity and growth decrease by one half for every 10 °C decrease in temperature below 35 °C (Li et al., 1999). In municipal wastewater plants, anaerobic treatment is carried out in the mesophilic ranges from 25 to 40 °C with an optimum temperature of approximately 35 °C (Parkinand Owen, 1986). Thermophilic anaerobic digestion has several advantages including higher rates of degradation in a small size digester requiring less capital cost, faster solid-liquid separation, and better control of bacterial and viral pathogens (Mackie and Bryant, 1995). In thermophilic temperature ranges, reaction rates precede at much higher rates than mesophilic ranges. Loading potentials of anaerobic bioreactors are significantly higher temperature (55 ^{o}C) (Dugba and Zhang, 1999). Even with these advantages, thermophilic wastewater treatments are not commonly applied. In comparison to mesophilic operational systems, thermophilic reactors require more energy for heating, produce poorer quality supernatant which contains larger quantities of dissolved solids, and have less process stability (Parkin and Owen, 1986; van Lier et al., 1996).

2-6-2. pH

The pH is one of the most important factors to be controlled in anaerobic process. Soluble pH impacts the structure of the microbial communities (Temudo et al., 2007). Each microbial group involved in anaerobic degradation has a specific pH range for optimal growth. The optimum pH range for specific hydrogen production rate is 5.5-5.7 (Van Ginkel et al., 2001; Khanal et al., 2004). Acidogenic bacteria produce organic acid, which tend to lower the pH of the anaerobic reactor. The optimal pH in terms of biohydrogen production is a range of 5.0-6.0 which probably favors the activity of hydrogenases in dark fermentation (Li and Fang, 2007).

In addition, the pattern of intermediate VFAs is different under variable pH conditions. Butyrate and acetate are the two main products in the fermentation process where butyrate is preferentially produced at low pHs. Butyrate and acetate pathways are favored at pH 4.5-6.0 while at neutral or higher pH conditions, ethanol and propionate accumulate (Yokoyama et al., 2007a). They showed that at low pH conditions (<6), the product spectrum consisted mainly of butyrate and acetate while at high pH, the spectrum shifted to acetate and ethanol. It is noteworthy that under both high and low pH conditions, the fermentation pattern was clearly associated with the dominance of *Clostridium* species, whereas at intermediate pHs, metabolic shifts involved higher microbial diversity (Temudo et al., 2008). This suggests that pH not only shifts the metabolic pathways but also results in major changes in microbial communities.

Table 2-1. Optimal pH for biohydrogen production according the organic substrate.

Substrate	Reactor	pH range	pH optimum	Reference
Rice bran	Batch	7	-	Noike and Mizuno, 2000
Cow waste slurry	Batch	6-7.5	7.0	Yokoyama et al., 2007b
Food waste	CSTR	5.0-6.0	5.5	Shin and Youn, 2005
Food waste	ASBR	5.3	-	Kim and Shin, 2008
Food waste	CSTR	5.5-6.0	-	Karlsson et al., 2008
Vegetable waste	CSTR	5.5-7	6.0-7.0	Lee et al., 2008

2-7. Anaerobic Microbial Consumption of H_2

Three groups of bacteria are known to interfere directly or indirectly, the diversion of biohydrogen potential from carbohydrates, i.e. the Sulfate-Reducing Bacteria (SRB), the Methane-Producing Bacteria (MPB), and the Homoacetogenic Bacteria (HAB).

2-7-1. Sulfate-Reducing Bacteria

According to theoretical thermodynamics, the most efficient biochemical reaction using hydrogen involves the sulfate/nitrate-reducing microorganisms, even at a low hydrogen concentration of only 0.02 ppm in the presence of sulfate or nitrate (Cord-Ruwisch et al., 1998). It has been shown that SRB have a thermodynamic advantage over MPB and HAB (Valdez-Vazquez and Poggi-Varaldo, 2009). Some waste especially from pulp/paper industry, sea-food processing, distilleries, edible oil and wet corn milling, contains high sulfate concentrations which perturb hydrogen anaerobic digestion as well as produce sulfide gas which is hazardous for fuel cells (Lin and Chen, 2006; Briones et al., 2009). Short HRTs are not sufficient to inhibit these microorganisms. Even at a HRT of 2 h, the interspecies transfer metabolites such as hydrogen, carbon dioxide and VFA, which are immediately consumed by SRB under sulfate-rich conditions (Valdez-Vazquez and Poggi-Varaldo, 2009). At longer HRT, hydrogen is converted either to methane with carbon dioxide by MPB under sulfate-limited

conditions, or to sulfidic acid by SRB if sulfate is abundant in the substrate (Mizuno et al., 1998). Along with the concentration of sulfate and HRT, pH is a key factor in sulfate reduction. pH values lower than 6 significantly inhibit the activity of SRB (Lin and Chen, 2006; Hwang et al., 2009).

2-7-2. Homoacetogenic Bacteria

Homoacetogenic bacteria are strictly anaerobic microorganisms which catalyze the formation of acetate from H_2 and CO_2. They possess special enzymes which catalyze the formation of acetyl-CoA that is converted either to acetate in catabolism or to cell carbon in anabolism. The homoacetogens are very versatile anaerobes, which convert a variety of different substrates to acetate as the major end product (Diekert and Wohlfarth, 1994). Thisimpliesthat in experimental studies the biohydrogen production measured might be lower than the expected calculated value due to accumulation of acetate (Antonopoulou et al., 2008). As no methane was detected in the biogas and the propionate mass balance did not explain hydrogen losses, hydrogen was assumed to be consumed by acetogenic bacteria (Kotsopoulos, 2009).

2-7-3. Hydrogen Production from Anaerobic Fermentation

In view of the transition to hydrogen as a major energy carrier in the future, new routes for hydrogen production need to be explored. The production of hydrogen from biomass is one of the options for contributing to the supply of exploitable renewable resources. Hydrogen can be produced from a vast range of biomass, using thermochemical, as well as fermentative, processes. Carbohydrates, such as sugars, starch or (hemi)cellulose, are the prime substrates for fermentative processes. For future sustainability of the energy supply, the utilization of (hemi)cellulose is of prime interest, as this component is most abundant in crops that can be grown for the purpose of energy supply. To date, many studies have been done on fermentative hydrogen production from pure sugars and from feedstocks, such as by-products from the agricultural and food industry, municipal waste, or wastewaters (Li and Fang, 2007). Several factors have been studied in the research to develop a sustainable anaerobic fermentation system to produce hydrogen. The motivation for this research has been the potential economic and environmental benefits that hydrogen could deliver. The bacterial culture utilized and anaerobic stages used to produce hydrogen have received renewed attention from researchers. Before large scale quantities of hydrogen can be produced these factors and others must be evaluated.

2-8. Two-Stage Anaerobic System

The two-stage system essentially comprises acidogenic and methanogenic processes. First in the acidogenic process, organic polymers, carbohydrates, proteins, and lipids are degraded to volatile fatty acids (VFAs), which are metabolized to methane in the subsequent methanogenic step. In order to realize a two-stage fermentation process comprising bothhydrogen and methane fermentation, a high-performance methanogenic reactor is required subsequent to the hydrogenogenic process.

Theoretically, up to 33% of the electrons in hexose sugars can go to H_2 when growth is neglected. This means at least 66% of the substrate electrons remain in the volatile fatty acid fermentation products. The addition of a methanogenic reactor in series after the hydrogen-producing reactor, as proposed by Hawkes et al. (2002), would allow for the conversion of the VFAs into methane, thereby increasing the amount of electrons recovered as renewable energy. Such a two-phase hydrogen-producing system is similar to two-phase anaerobic digestion where the acid-forming and methane-forming organisms are physically separated (Demirel and Yenigün, 2002). The difference between the two systems lies in the fact that the first reactor in two-phase hydrogen-producing system is optimized for H_2 production whereas the first stage of two-phase anaerobic digestion is not. The acid phase reactor of two-phase anaerobic digestion can range from fully methanogenic to having <1% methane in the biogas (Lee et al., 2009).

An important possible benefit of a two-phase hydrogen-producing system is that methanogenic treatment of the hydrogen reactor effluent will remove the VFAs, thereby recovering the alkalinity added for pH control. Therefore, recycling effluent from the methane phase to the hydrogen-producing phase should reduce the external alkali required for pH control in the hydrogen reactor.

2-9. References

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CHAPTER 3

Hydrogen Production from Sulfate- and Ferrous-Enriched Wastewater

Abstract

The quantitative relationship between sulfate reducing bacteria (SRB) and hydrogen (H_2) production from sulfate (SO_4^{2-}) and ferrous [Fe(II)] enriched wastewater was investigated. Both Fe(II) (0-11,600 mg/L) and SO_4^{2-} (0-20,000 mg/L) improved the H_2 production efficiency from wastewater. The H_2 yields were increased up to 1.9 mol H_2/mol glucose in 580-1750 mg Fe(II)/L and 1000-3000 mg SO_4^{2-}/L enriched wastewater at pH 5.8-6.2. Quantitative Fluorescence In Situ Hybridization (FISH) analyses revealed that the specific sulfate reducing activities (SSRA) were increased from 0.08 and 0.06 to 0.16 and 0.21 g TS/g SRB h in response to variations in sulfate concentration from 300-20,000 mg/L at pH 5.8 and 6.2, respectively. H_2 production was not influenced by low SSRA (≤0.1 g TS/g SRB h), which was independent of pH variation. The results demonstrated that the SSRA and Fe(II) concentration can significantly influence on the biological H_2 production from SO_4^{2-} and Fe(II) containing wastewater.

Key words: Biohydrogen, Quantitative SRB, SO_4^{2-}, Fe(II), pH

3-1. Introduction

Dark fermentation is a modified version of the classic anaerobic process to preferentially produce hydrogen (H_2), while suppressing methane production. Biological H_2 production utilizes organic wastewater or other wastes as raw materials, which contain a variety of organic substrates (Lin and Chen, 2006). Sulfate is a common constituent of many industrial wastewaters, such as an acid waste stream of a synthetic drug plant, yellow ginger processing wastewater, and the pulp/paper, seafood processing and alcohol fermentation industries. These wastewaters typically contain high sulfate concentrations (>20,000 mg/L) (O'Flaherty et al., 1998; Zhao et al, 2008). The high sulfate content in these wastes has been found to adversely affect anaerobic digestion, and hence, the fermentative H_2 production (Bitton 1994; Chen et al., 2008). Treatment of sulfate-containing wastewater by anaerobic fermentation results in sulfate reducing bacteria (SRB) proliferation (Zhao et al., 2008). The operational pH value is an important parameter in the anaerobic process, influencing on the activities of SRB and their metabolic pathways (Lopes et al., 2010). The fermentation process can cause a drop in the pH value in the fermentation reactor; thus base buffer is added to wastewater treatment plants to avoid excessive drops in pH (Romli et al., 1994). In a previous study, H_2 production and residual sulfate level were decreased with increasing pH from 5.5 to 6.2 (Hwang et al., 2009a). Another study showed that increased H_2 production at high sulfate concentrations (3000-5000 mg/L) and hydraulic retention time (HRT) of 6 h at pH 5.5 occurred due to increasing

concentrations of Fe(II), added to the reactor which improved the bioactivity of hydrogenases resulting in higher H_2 production. Therefore, proper sulfate, control and low pH are desirable for better performance of fermentation reactors. Little is known about sulfide toxicity for acidogens during the acidogenic phase while sulfate reduction is highly associated with the amount of H_2 production at short HRT (Liu et al., 2001). Sulfate is reduced to sulfite and then to sulfide by SRB, which can compete with H_2-producing acetogens (HPAs) for the same electron donors such as acetate and hydrogen. The competition leads to the H_2 transversals among the microbial species under anaerobic environment (Li and Fang, 1996). Fe(II) clusters play significant role in electron transfer between the Hcluster and the external electron carrier. Fe(II) not only plays an important role in increasing hydrogenase activity but also enhances the synthesis of H_2 contained cells (Adams, 1990). It can be suggested that processes were accelerated by improving the cell metabolism (Hwang et al., 2009b; Lee et al., 2009). The decreased H_2 production at pH 6, as compared with pH 5.5, was attributed to the increase of SRB activity, which competed against the activity of H_2-producing bacteria (HPB) (Hwang et al., 2009a), reflecting a complex interplay of various parameters, including SRB, COD/SO_4^{2-} ratios, Fe(II), SO_4^{2-} concentration and pH, in the fermentative reaction. COD/ SO_4^{2-} ratios is available for the effect of high sulfate concentrations on microbial hydrogen production (Lin and Chen, 2006; Hwang et al., 2009b). The relatively invariant hydrogen production with varying COD/SO_4^{2-} ratios was presumably due to low pH (5.5) at which the activity of SRB was significantly

suppressed (Hwang et al; 2009b). In order to obtain an appropriate fermentation system from sulfate and ferrous-enriched wastewater, it would be beneficial to quantitatively predict SRB activity during the H_2 fermentative process for optimizing substrate utilization in the H_2 reactor.

The objective of this work was to investigate the effects of SRB activity on fermentative H_2 production in synthetic wastewater enriched with sulfate and ferrous under controlled pH conditions (5.8-6.2). Fluorescence In Situ Hybridization (FISH) analysis was used to identify and monitor the changes in fermentative microbial populations and allow for quantitative interpretation of SRB in the bioreactor.

3-2. Materials and Methods

3-2-1. Seed Sludge

Sludge used in this study was collected from the anaerobic digesters of a municipal wastewater treatment plant (Water Supply and Drainage Center, South Korea). The pH, carbohydrate and volatile suspended solids (VSSs) were 6.8, 1.1, and 4.8 g/L, respectively, in the seed sludge. Glucose (15 g COD/L) was used as a substrate. Iron sulfate ($FeSO_4 \cdot 7H_2O$) solution was used as the iron and sulfate source with concentrations ranging from 0 to 11600 mg Fe(II)/L and 0 to 20000 mg SO_4^{2-}/L. Control experiments (without the addition of $FeSO_4 \cdot 7H_2O$) were also conducted. Volatile fatty acids (VFAs) and alkalinity of the sludge were 410 and 2180 mg/L, respectively. The sludge was acclimatized to a synthetic medium (glucose 10 g/L) for 1 month in an anaerobic chemostat reactor. Synthetic medium and the acclimated sludge was mixed in 1:1 ratio (v/v) and added to 4-L reactor, which was operated in chemostat mode at 35 ^{o}C with hydraulic retention time (HRT) of 12 h for one month. The mixture was continuously fed to the reactor using a micro-tube pump (EYELA, MP-3, Japan). Prior to cultivation, the reactor was flushed with N_2 gas for 15 min to ensure anaerobic conditions.

3-2-2. Medium Composition and Experimental Batch Setup

The medium used for H_2 fermentation consisted of glucose as the sole carbon substrate (Hwang et al., 2009b). The medium was prepared daily and stored in a substrate reservoir maintained at 4±1 °C. Hydrogen production experiments were

performed using sulfate and ferrous enriched synthetic wastewater. The experiment was carried out in triplicate using 120 mL serum bottles with a working volume of 80 mL. The inoculum from the 4-L fermenter in chemostat mode and the synthetic wastewater were transferred into a serum bottle (40 mL) in an anaerobic chamber with the flushing of N_2 gas. The serum bottle was sealed tightly with a butyl rubber stopper and an aluminum cap. The pH was adjusted using 1N HCl and 1N NaOH. The bottles were placed in a water bath shaker at 120 rpm and 35 °C during the experiment.

3-2-3. Analyses

Mixed liquor volatile suspended solids (VSS) were analyzed according to standard methods (APHA, 1995). The H_2 gas was analyzed by a gas chromatograph, equipped with a thermal conductivity detector (Shimadzu GC-14, Japan), using a stainless steel column packed with molecular sieve 5A and Ar as a carrier gas. The operational temperatures of the injector, detector, and column were 80, 110 and 60 ^{o}C, respectively. CO_2, CH_4, and H_2S were measured by the same detector, using a 2.5 m stainless column packed with Hayesep Q (80/100) and helium (He) as a carrier gas. Ethanol and VFAs were analyzed by a gas chromatograph (GC-8A, Shimadzu Co., Japan) equipped with a flame ionization detector and a glass column packed with 10% REOPLEX 400. The temperatures of the injector port, detector, and column were 240, 240, and 140 ^{o}C, respectively. The pH was measured by a pH meter (Thermo Orion 290A, Orion Corporation).

Sulfate concentration was analyzed by an ion chromatography (DX-120, Dionex Corporation, USA) equipped with an analytical column (Dionex Ionpac AS14-4 mm) and a guard column (Dionex Ionpac AG14-4 mm) after pretreatment with 0.45 mm GF/C (Glass fiber filter, Type C). Total sulfide (TS) was determined by iodometric methods (APHA, 1995). Carbohydrate concentration was analyzed using the anthrone-H_2SO_4 method (Mah et al., 1990). Cumulative H_2 production was obtained over the course of the batch experiment, and was analyzed using the modified Gompertz equation (Lay et al., 1999; Van et al., 2001; Logan et al., 2002).

SRB concentration was calculated as:

Total microbial community (mg/L) × quantitative SRB (%) (1)

The specific sulfate reducing activities (SSRA) was calculated from the total sulfide concentration and SRB concentration.

For quantitative analysis of the FISH images, specific probe hybridized cell areas were measured by scanning the FISH images, and then used for the analyses of the proportions of *Desulfovibrio* sp. and *Desulfobulbus* sp. relative to the total bacteria specifically hybridized to an Eubmix probe. The average fraction was determined from about 10 confocal laser scanning microscopy (CLSM) images from image-processing software (IMT i-Solution, version 3.0). The FISH technique with 16S rRNA-targeted oligonucleotide probes was employed to

monitor the changes in the microbial community in the batch reactor system (Amann, 1995). Oligonucleotides were synthesized and fluorescently labeled with a hydrophilic sulfoindocyanine dye (Cy-3) and/or fluorescein isothiocyanate (FITC) and/or 6-carboxy-fluorescein (6-FAM) at the 5´ end (Bionics, Seoul, South Korea). The oligonucleotide probes used in this study are specific for phylogenetic groups of HPB and SRB, as listed in Table 3-1. Anaerobic samples were recovered from the reactor and fixed in 4% freshly prepared paraformaldehyde solution for 3-4 h at 4 ^{o}C. Phosphate-buffered saline (PBS) was used to rinse the anaerobic samples. Each fixed sample was placed in a small aluminum cup overnight at room temperature to allow the Jung OCT compound (Leica Int., Germany) to penetrate the anaerobic samples. Anaerobic samples were frozen at -20 ^{o}C and coated with 0.1% gelatin in the presence of 0.01% chromium potassium sulfate and dried overnight at room temperature (25 ^{o}C). The specimens were dehydrated by successive 50, 80, and 98% ethanol washes (3 min each) followed by air drying and stored at room temperature until further use. In situ hybridization was performed in a hybridization incubator using formamide at 46 ^{o}C for 2 h. Hybridization was followed twice by a stringent washing step at 48 ^{o}C for 10 min with 50 mL pre-warmed washing buffer (20 mM Tris hydrochloride [pH 7.2] and 0.01% sodium dodecyl sulfate). The washing buffer was removed by rinsing the slides with distilled water, and the slides were air dried. The slides were mounted to avoid bleaching and examined with an Axioplan epifluorescence microscope

(Carl Zeiss) and an MRC-1024 (Biorad, U.K.) CLSM equipped with Kr/Ar lasers (excitation wave length 494 nm and 650 nm) and HeNe lasers (550 nm).

Table 3-1. Characteristics of the 16S rRNA-directed oligonucleotide probes used for FISH analysis

Probe	Specificity	Probe sequence (5'-3')	Dye
Csac67	*Clostridium* spp.	CTCGGACATTACTGCCCGCG	Cy-3
SRB687	*Desulfovibrio* spp.	TACGGATTTCACTCCT	FITC
SRB660	*Desulfobulbus* spp.	GAATTCCACTTTCCCCTCTG	6-FAM
EUB338 I	Bacteria	GCTGCCTCCCGTAGGAGT	
EUB338 II	Bacteria not covered by EUB338 I and EUB338 III	GCAGCCACCCGTAGGTGT	Cy-3
EUB338 III	Bacteria not covered by EUB338 I and EUB338 II	GCTGCCACCCGTAGGTGT	

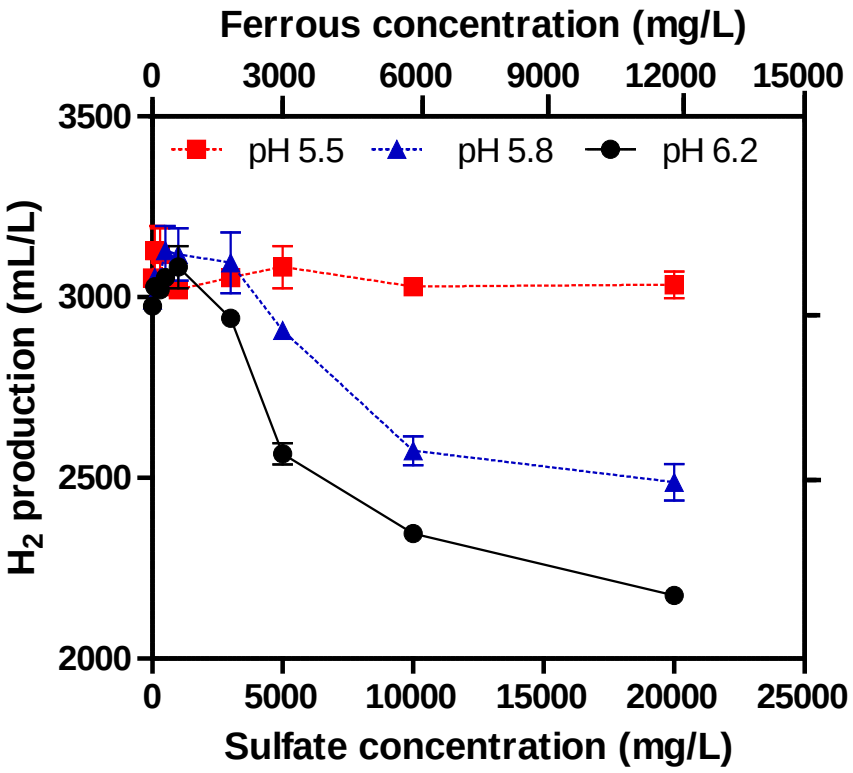
3-3. Results and Discussion

3-3-1. Effect of SO_4^{2-}, Fe(II), and pH on H_2 production

Hydrogen production and SRB growth at pH values (5.8 and 6.2) and different concentration of SO_4^{2-} (0-20000 mg/L) and Fe(II) (0-11600 mg/L) during H_2 fermentation process are shown in Fig. 3-1. H_2 production was observed at lower pH conditions (e.g., pH 5.5), irrespective of the variation in SO_4^{2-} concentration. The maximum H_2 production (3300 mL/L) was observed at pH 5.8, and 3000 mg SO_4^{2-}/L, while the minimum H_2 production (2175 mL/L) was obtained at pH 6.2 and 20000 mg SO_4^{2-}/L. Declining H_2 production was attributed to the gradual acclimation of microflora to the sulfate reducing environment in response to increased sulfide levels (Hwang et al., 2009b). H_2 production increased from 2964 to 3262 mL/L when the sulfate concentration was increased from 0 to 1000 mg/L at pH 5.8. The marked increase in H_2 production might also be due to the simultaneous increase of Fe(II) concentration (2900 mg/L) at high sulfate concentrations (1000 mg/L). Iron was especially beneficial for bacterial growth, but an excess amount of Fe(II) (ca. 3000 mg $FeSO_4$/L) could be unfavorable for microbial activity, leading to reduced H_2 production (Zhang et al., 2005). An inhibitory mechanism involving iron oxyhydroxide coatings on a cell surface was also reported by Liu et al. (2001), who observed the formation of iron mineral coatings on *Shewanella putrefaciens* after the exposure of cells to Fe(II). Growth rates of SRB were negligible in sulfate concentrations under acidic condition (pH 4-6), indicating that their growth was not evident at pH lower than 6 (Fortin et al.,

1996). This result implies that SRB could not survive and active at low pH where H_2-producing bacteria was dominated. It was also observed that the increase in pH at varying SRB growth rates influenced H_2 yield and H_2 content in the biogas (Hwang et al., 2009a).

(A)



(B)

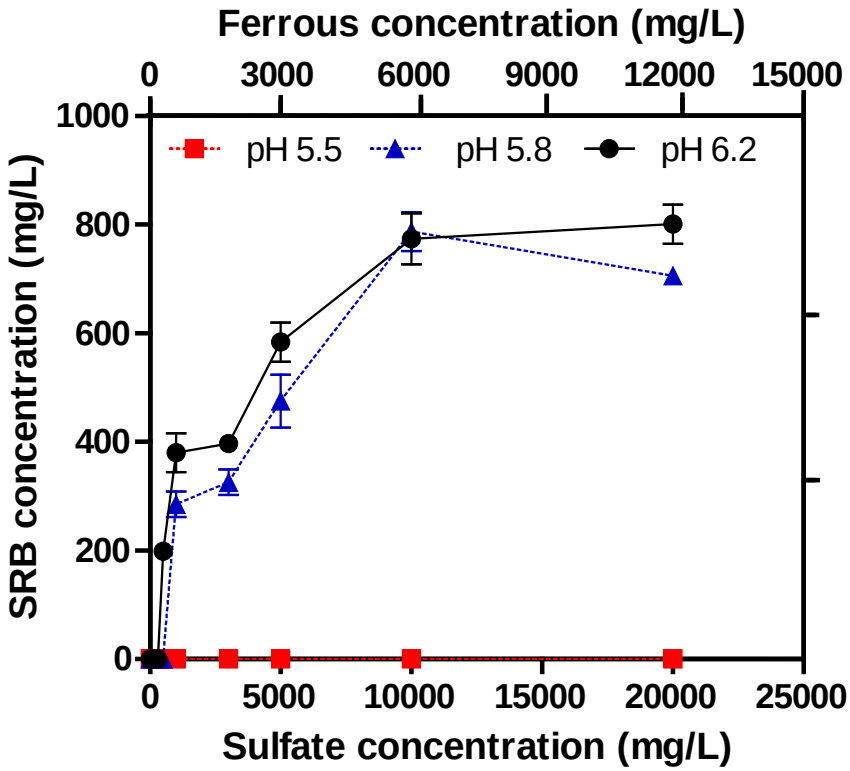


Figure 3-1. Relationship between (A) H_2 production and (B) SRB concentration at various pHs and sulfate concentrations.

The H_2 yield, H_{max}, and gas composition under varying pH and sulfate concentrations are shown in Table3-2 and Fig. 3-2. The average H_2 yield at 1000 mg SO_4^{2-}/L was 1.9 and 1.8 mol H_2/mol glucose at pH 5.8 and 6.2, respectively (Table 3-2). The H_2 content was 51, 48 and 46% at pH 5.5, 5.8 and 6.2, respectively. The H_2 content reached a maximum of 53 and 52% when sulfate concentration varied between 1000 and 3000 mg/L at pH 5.5. High sulfate concentrations up to 20000 mg/L at pH 5.5 did not affect H_2 production, suggesting that SRB in the culture were not active at such conditions, and H_2 production was not influenced by high sulfate concentrations (Chen et al., 2008; Hwang et al., 2009). However, H_2 content (51 and 50%) was maintained relatively constant at pH 5.8 and 6.2, irrespective of the SRB community, up to 400-510 mg/L at various sulfate concentrations (500-1000 mg/L). Similar results have been observed where SRB metabolic activities did not inhibit H_2 production in a fermentation reactor with concentrations under 30 mg/L of dissolved sulfide (Byrant et al., 1977; Lee et al., 2009).

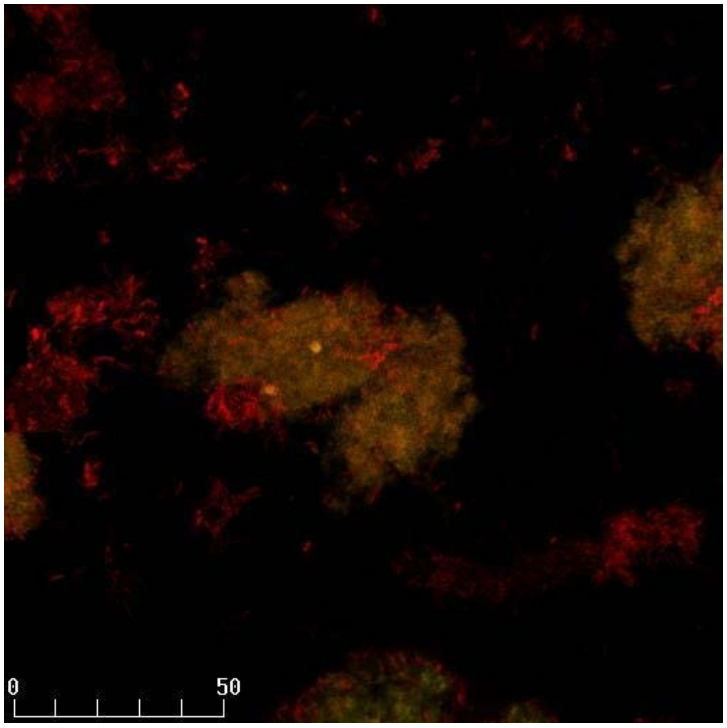
Table 3-2. Summary of operational performances obtained from wastewater by varying pH, sulfate and iron concentrations.

$FeSO_4$ (mg/L)		pH	H_2 yield (mol H_2/mol glucose)	H_2 content (%)	*H (t)* (mL/L)	R_{max} (mL/L h)	λ (h)
SO_4^{2-}	Fe(II)						
0	0		1.8	50	3012.5	91.9	6
100	58		1.7	49	3075.4	85.6	6
300	175		1.8	50	3084.8	85.3	6
500	290		1.7	49	3262.5	84.5	6
1000	580	5.8	1.9	51	2980.3	77.2	6
3000	1750		1.9	48	3262.4	75.2	6
5000	2900		1.7	47	2887.5	72.2	6
10000	5800		1.5	44	2587.4	68.9	8
20000	11600		1.4	44	2450.2	68.9	8
0	0		1.7	49	2962.5	92.9	6
100	58		1.8	49	3012.4	88.8	6
300	175		1.7	49	2987.5	82.2	6
500	290		1.8	50	3012.4	76.7	6
1000	580	6.2	1.9	49	3025.1	74.2	6
3000	1750		1.8	48	2925.0	71.7	6
5000	2900		1.6	45	2537.5	69.2	7
10000	5800		1.5	42	2350.6	64.9	7
20000	11600		1.4	41	2175.2	61.9	8

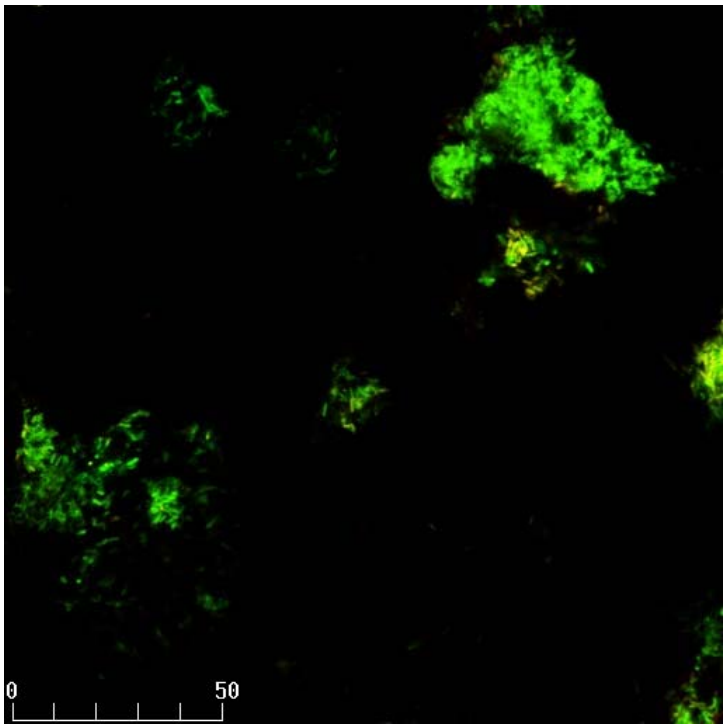
3-3-2. FISH image analysis of HPB and SRB

The FISH images of fermentative microorganisms in the anaerobic reactor are shown in Fig. 3-2. The presence of *Clostridium* sp., *Clostridium butyricum*, *Clostridium perfringens* and *Ruminococcus flavefaciens* as HPB were detected under all experimental conditions. The FISH image showed that *Clostridium* sp. and *Desulfovibrio* sp. accounted for 42% and 14% of the total microflora, respectively, at pH 5.8 and a sulfate concentration of 1000 mg/L, based on the Eubmix calculation (see Fig. 3-2). The hybridization images revealed that the SRB members were increased from ca. 5% to 17% in wastewater with the increase of ferrous ion concentrations (580-11600 mg/L). FISH images showed the ca. 14% increase in the SRB quantity by enriching the sulfate concentration at pH 5.8. Quantitatively, the SRB (*Desulfobulbus* and *Desulfovibrio*) in total microbial community was remaining constant between 10-16% at the pH 5.8-6.2 even though the sulfate concentration increased. The FISH images revealed that the increase of the feed pH and sulfate concentration leads to increasing the SRB quantity which subsequently consumed more COD and caused the drop in H_2 production. This was one of the critical features indicative of a typical acidogenic sulfate reducing reactor, because HSRB acts as a consumer of hydrogen (Ren et al., 2007). This is probably due to the fact that SRB tend to grow in small "pockets" at elevated pHs. This unfavorable condition did not encourage the growth of SRB, despite the high sulfate concentration.

(A)



(B)



(C)

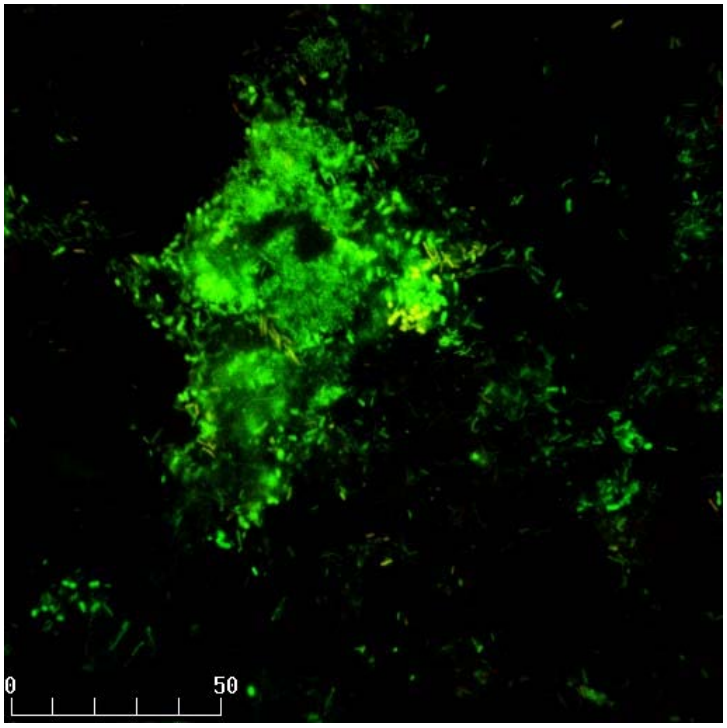


Figure 3-2. Fluorescence *in situ* hybridization images of *Clostridium* spp., *Desulfovibrio* sp. and *Desulfobulbus* sp. A) Casc 67 (Cy3, red), EUBmix (Cy3, FITC, green), B) SRB557 (FITC, green), EUBmix (Cy3, red) and C) SRB 660 (6-FAM, green), EUB mix (Cy3, red) at pH 5.8 and sulfate concentration 1000 mg/L, scale bars within each panel indicate distance in microns (μm).

Therefore, SRB cells were not detected by FISH analysis at pH 5.5. pH increase from 5.5 to 5.8 and to 6.2 lead to an increase of SRB fractions, with an average of 0, 10 and 16%, respectively, at 5000 mg SO_4^{2-}/L. The marked increase in pH at sulfate concentrations from 1000 to 5000 mg/L might be also due to the simultaneous increase of SRB. The remaining fraction of the SRB community in the fermentation reactor is believed to be heterotrophs, which likely offered the potential for sulfate reduction supported by organic carbon (Turick et al., 2002).

3-3-3. Effect of Sulfate Reduction on the Activities of SRB in the H_2 Fermentation Reactor

Specific sulfate reducing activities of *Desulfovibrio* sp. and *Desulfobulbus* sp. were calculated by multiplying MLVSS with the fraction of SRB found in the total bacteria from the quantitative FISH image analyses (Fig. 3-3 and Table 3-3). The SSRA were plotted against the ratio of *Desulfovibrio* sp. to the total amount of SRB (*Desulfovibrio* sp. and *Desulfobulbus* sp.). Specific sulfate-reducing activities increased as the *Desulfovibrio* ratio (1-*Desulfobulbus* sp.) increased.

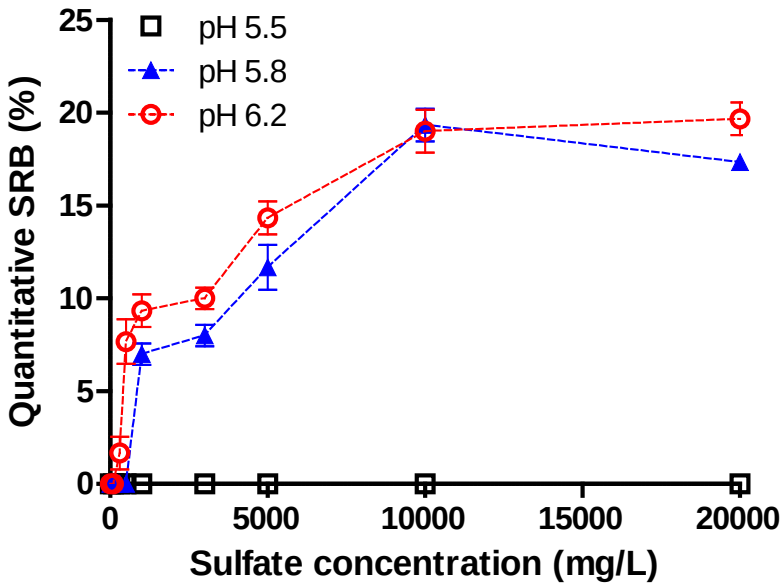


Figure 3-3. Quantitative SRB contents based on total bacteria (Eubmix) from sulfate and ferrous enriched wastewater

Significant variations of SSRA (0.06-0.21 g TS/g SRB h) were not observed under all experimental conditions. However, specific sulfate reduction did not influence H_2 production to a great extent at sulfate concentrations from 1000 to 5000 mg/L and from 500 to 3000 mg/L at pH 5.8 and 6.2, respectively. Further, regardless of sulfate concentrations, there was a little difference in the specific activity, with an average of 0.07 g TS/g SRB h at pH 6.2 and with sulfate concentrations of 300-3000 mg/L. This might be attributed to the Fe(II) concentration added to the reactor (Amann, 1995). The competitive relationship between microbial Fe and SO_4^{2-} reduction is presumed to prevail and that Fe reduction may deplete any available substrate below the threshold level for utilization by sulfate reduction when sufficient Fe(III) is available (Lovley and Phillips, 1987; Lovley and Goodwin, 1998). The relatively invariant H_2 production with a varying SRB community was presumably due to a specific condition (≤0.1 g TS/g SRB h), for which the activity of the SRB was significantly suppressed. The presence of SRB with high specific activity (>0.1 g TS/g SRB h) influenced H_2 production. This observation is confirmed by the H_2 content in our study.

Table 3-3. Quantification of SRB using FISH analysis and batch kinetics analysis

$FeSO_4$ (mg/L)		pH	*Desulfovibrio* sp.[a] (%)	*Desulfobulbus* sp. [a] (%)	*Desulfovibrio* ratio	Specific activity (g TS/g SRB h)
SO_4^{2-}	Fe(II)					
0	0	5.8	N.D.	N.D.	0	0
100	58		N.D.	N.D.	0	0
300	175		N.D.	N.D.	0	0
500	290		N.D.	N.D.	0	0
1000	580		3.3	2.0	0.40	0.08
3000	1750		4.2	1.9	0.54	0.09
5000	2900		8.7	2.9	0.67	0.09
10000	5800		14.6	4.1	0.72	0.13
20000	11600		15.4	2.6	0.78	0.16
0	0	6.2	N.D.	N.D.	0	0
100	58		N.D.	N.D.	0	0
300	175		1.3	0.7	0.46	0.06
500	290		3.1	1.5	0.51	0.07
1000	580		6.2	2.6	0.58	0.07
3000	1750		8.3	3.2	0.61	0.10
5000	2900		11.7	4.3	0.63	0.17
10000	5800		13.1	3.9	0.70	0.18
20000	11600		16.5	3.1	0.81	0.21

N.D.: Not detected; [a] Percentage of the microbe relevant to all bacteria (Eubmix)

3-4. Conclusions

The presence of SRB had little influence on H_2 production (1.7-1.9 mol H_2/mol glucose) at SSRA (0.1 g TS/g SRB h) or with the variations in pH (5.8-6.2). Increased H_2 yield (1.9 mol H_2/mol glucose) occurred due to the presence of 580-1750 Fe(II)/L added to the reactor, which enhanced the activity of hydrogenases for higher H_2 production. Biohydrogen fermentation could be successfully operated in wastewaters enriched with high concentrations of sulfate and ferrous ions, with little variance in H_2 yield (1.6-1.7 mol H_2/mol glucose), whereas more variance in sulfate reducing activity.

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CHAPTER 4

Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system

Abstract

Anaerobic fermentation for hydrogen (H_2) production was studied in a two-stage fermentation system fed with different ripened fruit feedstocks (apple, pear, and grape). Among the feedstocks, ripened apple was the most efficient substrate for cumulative H_2 production (538 mL) with a maximum H_2 yield (243.4 mL/g VS) in the first stage at a hydraulic retention time (HRT) of 18 h. The additional cumulative biohydrogen (402 mL) was produced in the second stage with the reused residual substrate from the first stage. The major byproducts in this study were butyrate, acetate, and ethanol, and butyrate was dominant among them in all test runs. During the two-stage system, the energy efficiency (H_2 conversion) obtained from mixed ripened fruits (RF) increased from 4.6% (in the first stage) to 15.5% (in the second stage), which indicated the energy efficiency can be improved by combined hydrogen production process. The RF could be used as substrates for biohydrogen fermentation in a two-stage (dark/dark) fermentation system.

Key words: Biohydrogen, Two-stage fermentation, Energy efficiency, RF, HRT

4-1. Introduction

Hydrogen (H_2) gas is a clean energy carrier that has been recognized as the most promising alternative to fossil fuels (Suzuki, 1982). H_2 has high energy content (122 kJ/g) and is extensively used in a wide range of energy and industrial applications. Approximately 96% of H_2 consumed globally is produced from fossil fuels via steam reformation of natural gas, partial oxidation of fuel oil, and gasification of coal (Ewan and Allen, 2005). These processes are highly energy-intensive and not always environmentally friendly, which may outweigh the advantages of a H_2-based economy (Nath and Das, 2004). There is a vital need for the development of cost-effective and environmentally friendly technologies for H_2 production from renewable resources. Anaerobic biohydrogen fermentation from organic wastes allows clean energy generation while reducing wastes (Zhu et al., 2008). Biological H_2 production provides a wide range of pathways to produce hydrogen, including direct or indirect bio-photolysis, photo-fermentation and dark-fermentation (Lin and Chang, 2004).

Dark fermentation from organic wastes is a promising and ecofriendly hydrogen production method (Benemann, 1996), and two-stage fermentation processes for hydrogen and methane production have been evaluated (Ting and Lee, 2007; Ueno et al., 2007b; Lee et al., 2010). Two-step dark/photo-fermentation systems have also been studied (Nath and Das, 2009; Liu et al., 2010). H_2 is an important intermediate in the microbial degradation of organic material in anaerobic environments (Schink, 1997), and a variety of pure or mixed substrates can be

utilized, including glucose, sucrose, starch, cellulose and waste materials containing these saccharides (Mizuno et al., 2000; Hussy et al., 2003). The efficiency of hydrogen production is affected by many factors such as type of inoculum, substrate composition, pH, organic loading and HRT (Kim et al., 2004; Liu et al., 2006; Li and Fang, 2007). These factors influence bacterial growth, fermentative pathways and bacterial communities, and they determine the overall hydrogen production (Zhu et al., 2008).

Anaerobic digestion is applied to a wide range of carbohydrate rich feedstocks including food industry wastes (Han and Shin, 2004), household solid waste (Liu et al., 2006), mixtures of pulverized garbage and shredded paper wastes (Ueno et al., 2007a), artificial organic solid waste (Ueno et al., 2007b) and wastewater sludge (Ting and Lee, 2007). Food waste has proven to be highly desirable substrate for anaerobic fermentation due to its high digestibility and well balanced carbon and nutrient contents (Zhang et al., 2007). The fermentative bacteria can utilize carbohydrate in the dark fermentation process but the process remains effective only at substrate concentration below 35 g COD/L, which implied that microbial activity was inhibited in high substrate condition (>35 g COD/L) and resulted in limited H_2 production and carbohydrate degradation (Kim et al., 2006). The efficiency of the fermentation process depends on the food to microorganism ratio and hydrogen production is highly variable depending on this ratio (Pan et al., 2008). To our knowledge the operation of a two-stage (dark/dark) fermentation system for H_2 production from different RF has not been reported. In order to

select and design an appropriate two-stage (dark/dark) fermentation system, it would be beneficial to predict H_2 production during the two-stage fermentative process not only for the purpose of substrate reutilization in the second stage but also for optimizing the combined system.

The objectives of this work were (i) to determine the potential use of re-utilized RF from first stage for H_2 production using second stage fermentative reactors, (ii) to determine whether sewage sludge can reduce the alkalinity concentration required for pH control in the hydrogen reactor, and (iii) to investigate the effectiveness of a two-stage fermentation system for increased hydrogen recovery from different RF with relatively short HRT ($\leq$28 h).

4-2. Materials and Methods

4-2-1. Feedstock sampling and preparation

Rotten fruits waste was collected from a fruit shop in Wonju, S. Korea. Slurries of RFW were prepared by separately grinding 3 g of apple, 3 g of pear and 1.2 g of grapes in a blender using 100 mL of deionized water to facilitate grinding. Rotten fruit waste slurries were sealed in plastic bags, stored in a refrigerator at 4^{o}C to reduce deterioration by acidification, and warmed to room temperature (25^{o}C) prior to testing. Characteristics of the ground RFWs are shown in Table 4-1.

Table 4-1. Characteristics of RFW-slurry, corn powder, GFBM and SL

Parameter	Apple	Pear	Grape	Mixed (AP)[a]	Mixed (APG)[b]	Corn powder	GFBM[c]	SL[d]
Carbohydrate (g/L)	45.9 (0.9)	64.5 (1.7)	73.5 (4.2)	51.3 (1.1)	66.9 (3.1)	31.4 (0.2)	0.04 (0.0)	1.4 (0.1)
COD (g/L)	132.2 (2.3)	189.1 (1.4)	178.6 (3.2)	142.1 (2.2)	177.5 (4.2)	49.5 (0.7)	0.4 (0.2)	9.7 (0.1)
Total solids (g/L)	4.8 (0.1)	5.1 (0.1)	5.6 (0.2)	4.3 (0.1)	4.6 (0.1)	4.2 (0.1)	0.1 (0.0)	5.2 (0.2)
Total protein (g/L)	0.9 (0.03)	0.8 (0.04)	1.2 (0.02)	0.7 (0.04)	0.9 (0.01)	0.5 (0.01)	0.01 (0.0)	0.2 (0.02)
VFA (g/L)	3.3 (0.2)	3.5 (0.1)	1.3 (0.2)	3.3 (0.1)	3.2 (0.2)	2.6 (0.1)	0.2 (0.1)	0.4 (0.1)
pH	5.8 (0.1)	5.6 (0.1)	5.7 (0.1)	5.6 (0.1)	5.8 (0.1)	6.2 (0.1)	7.1 (0.1)	6.7 (0.1)

Numbers in parenthesis represent standard deviation.
[a] Apple:Pear; [b] Apple:Pear:Grape; [c] Glucose free basal medium and [d] Seed sludge

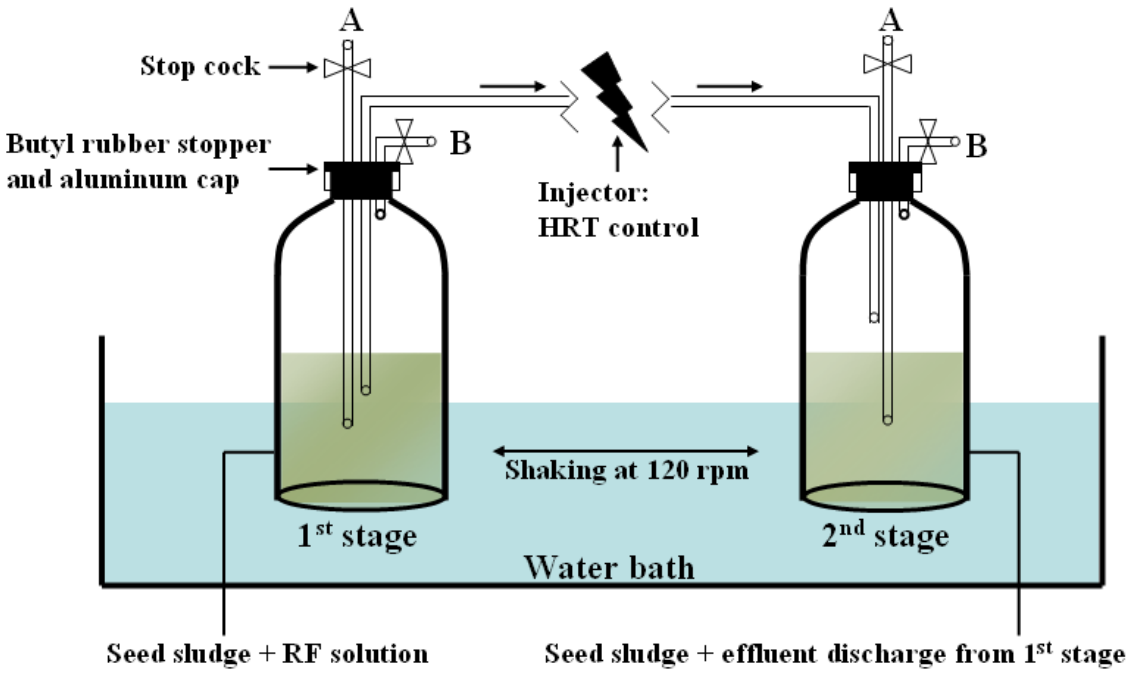
4-2-2. Seed microorganisms

Anaerobic hydrogen-producing mixed microbial communities were enriched at the start of the study. The sewage sludge (SL) used in this study was collected from the anaerobic digesters of a municipal wastewater treatment plant (Water Supply and Drainage Center, S. Korea). The characteristics of the SL are shown in Table 4-1. To prepare hydrogen-producing bacteria, the sludge was boiled for 30 min to inactivate any methanogenic bacteria. Anaerobic spore-forming bacteria such as genera *Bacillus* and *Clostridium*, which can germinate back to an active vegetative state in favorable growth environments, were harvested (Rittmann and McCarty, 2002). Hydrogen-producing inocula were enriched in the reactor using preheated digested sludge as the original source of organisms and glucose basal medium as the substrate. The glucose medium contained the following constituents (in g/L): glucose, 10; NH_4HCO_3, 4.7; K_2HPO_4, 0.12; $MgCl_2 \cdot 6H_2O$, 0.1; $MnSO_4 \cdot 6H_2O$, 0.015; $FeSO_4 \cdot 7H_2O$, 0.025; $CuSO_4 \cdot 5H_2O$, 0.005; $CoCl_2 \cdot 5H_2O$, 0.0001, and $NaHCO_3$, 2. The substrates were prepared daily and stored in a substrate reservoir maintained at 4±1°C. The chemical oxygen demand (COD) of this feed solution was measured over the duration of the experiment to be 10.6±0.4 g/L. Experiments were conducted in triplicate. Anaerobic reactors of 4.0L capacity with 2.0 L working volume were used. A glucose medium volume of 1 L and 1 L of digested sludge were added to the reactor, which was operated in chemostat mode at 35^{o}C with HRTs of 8, 18 and 28 h for one month. Substrate (glucose) was continuously added into the reactor with a micro-tube pump

(EYELA, MP-3). Prior to cultivation, the reactors were flushed with N_2 gas for 15 min to ensure anaerobic conditions.

4-2-3. Experimental batch setup

Anaerobic digestion for hydrogen production was carried out using RFWs (apple, pear, and grape) individually or as a mixture. The two-stage fermentative system was developed for efficient conversion of various organic wastes into biogas energy. A schematic diagram of the two stage system is shown in Fig. 4-1. The RFWs were digested in the batch fermentative reactors to produce organic acids and biogas. After effluent discharged (100 mL) from the first stage fermentation was loaded into second reactor, the anaerobic microorganisms and residual carbohydrate reuse from the previous batch as well as the microorganisms transferred from the second fermentative reactor was used as inoculum. In the same manner, glucose-free basal medium (GFBM) and corn powder were used as control feedstocks in the experiment. The headspace of each bottle was flushed with N_2 gas and sealed tightly with a butyl rubber bung and an aluminum crimp. Bottles were placed in a water bath shaker at 120 rpm and 35°C for four days.



A: Sample port (liquid), B: Sample port (gas)

Figure 4-1. Schematic diagram of the two-stage fermentation system.

4-2-4. Analytical procedures

Soluble COD, total solids, and volatile suspended solids were measured according to standard methods (APHA, 1995). Protein and carbohydrate content were measured by the Lowry method (Anderson et al., 1982) and anthrone-H_2SO_4 (Mah et al., 1990), respectively. Samples were filtered with 0.45 mm GF/C (Glassfiber filter, Type C) filters. Solution pH was measured with pH meter (Thermo Orion 290A, Orion Corporation), and VFAs were analyzed using gas chromatograph (Shimadzu GC-8A, Japan) equipped with a flame ionization detector. Biogas in the vessel headspaces was periodically measured using gas chromatograph (Shimadzu GC-14, Japan) equipped with a thermal conductivity detector and a molecular sieve 5A (80/100 6 ft×1/8 ft) column with Ar as a carrier gas. The temperatures of the injector, detector, and column were maintained at 80, 110 and 60 °C, respectively.

4-2-5. Data analysis

Cumulative hydrogen production curves were obtained over the course of the batch experiment and were analyzed using the modified Gompertz equation (Lay et al., 1999):

$$H(t) = H_{\max} \exp\left\{-\exp\left[\frac{R_{\max} * e}{H_{\max}}(\lambda - t) + 1\right]\right\} \qquad (1)$$

where, $H(t)$ (mL) is the amount of hydrogen produced at time, H_{max} (mL) is the total amount of hydrogen produced, R_{max} (mL/h) is the biogas production rate, λ (h) is the lag phase, and e=2.71828. Constants were obtained as previously described (Van Ginkel et al., 2001).

Hydrogen gas production was calculated from the headspace measurements of the gas composition and the total volume of biogas produced at each time interval using Eq. (2):

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_H(C_{H,i} - C_{H,i-1}) \quad (2)$$

where, $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes at the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volumes at the current and previous time intervals, $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspaces of the bottles according to gas chromatography at the current and previous time intervals, respectively, and V_H is the total volume of headspace in the reactor (Logan et al., 2002).

4-3. Results and discussion

4-3-1. Characteristics of the different feedstocks

The characteristics of different feedstocks including glucose-free basal medium (GFBM), corn powder, and SL are summarized in Table 4-1. Slightly acidic pH values ranged from 5.6 to 6.2 were observed for RFWs and corn powder. The RFWs in this study contained soluble carbohydrate concentrations as high as 73.5±4.2 g/L for grapes, 64.5±1.7 g/L for pears, and 45.9±0.9 g/L for apples, while SL, GFBM, and corn powder contained soluble carbohydrates at significantly lower levels. Compared to RFWs, the SL inoculum which contains glucose basal medium as a substrate had a highly alkaline concentration. The solution pH and carbohydrate contents of RFW were favorable for hydrogen production because carbohydrates are suitable substrates for hydrogen-producing bacteria (HPB) (Motonobu et al., 2004). The GFBM was considered an unlikely substrate for hydrogen production on the basis of its low carbohydrate content (see Table 4-1).

Average concentrations of total solids (TS) for apples, pears and grapes were 4.8±0.1, 5.1±0.1, and 5.6±0.2 g/L, and the total VFAs of the apples, pears and grapes were 3.3±0.2, 3.5±0.1, and 1.3±0.2 g/L, respectively, indicating the presence of abundant amount of readily digestible organic contents. The volatile fractions of SL and GFBM were lower than those of the RFWs, implying larger indigestible fractions in those feedstocks which are consistent with the previous observation where the majority of volatile fraction of the SL is proteinaceous

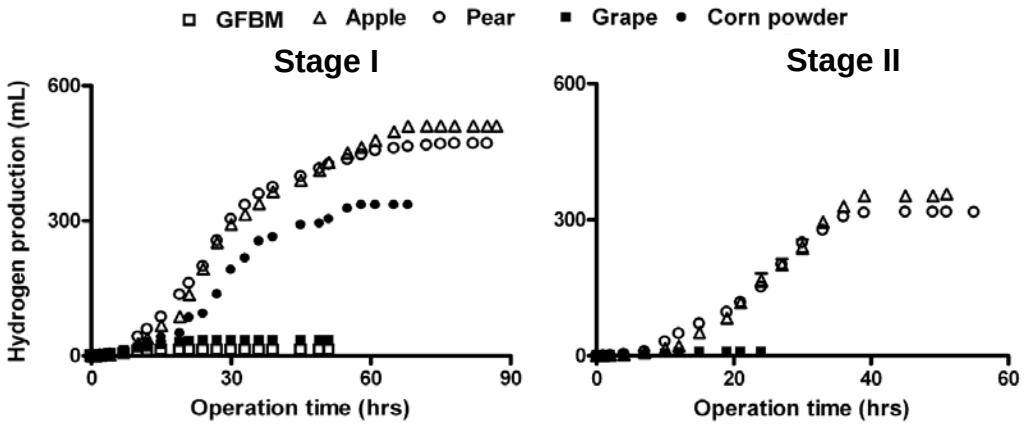
material, and the proteinaceous COD is more than 50% of the total COD in the SL (Hwang et al., 2009). In general, the RFW had a complementary nutrient composition and pH buffering capacity to those of the GFBM and SL, which suggested the potential for co-digestion during hydrogen production (Han and Shin, 2004).

4.3.2. Hydrogen production from different feedstocks

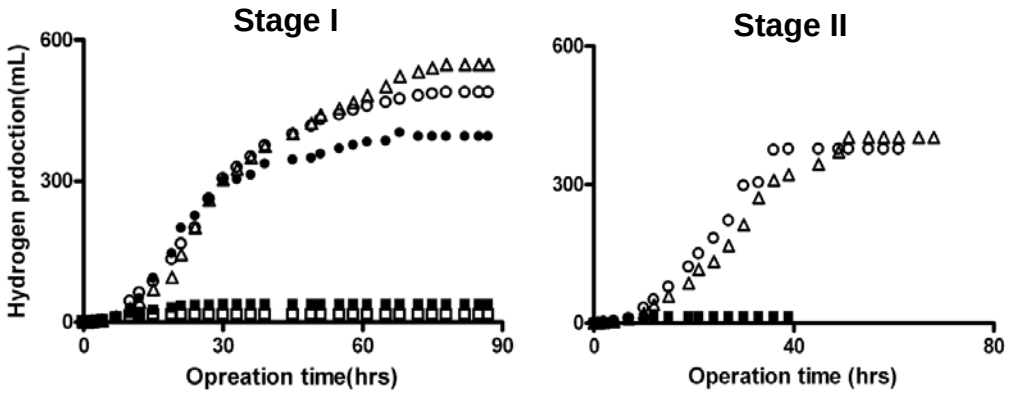
Batch fermentation for hydrogen production was conducted for 86 h. After first stage, the effluent was loaded to the second reactor by a graduated cylinder (see the Fig. 4-1). The maximum amount of hydrogen productions (H_{max}= 538 mL) was obtained from apple in the first stage at HRT 18 h (Fig. 4-2). The H_{max} decreased from 538 to 402 mL for apple, and it also decreased from 488.3 to 376.5 mL for pear when the feedstocks were transferred from the first stage to the second stage at HRT 18 h. With GFBM and grape substrates, a low amount of hydrogen was obtained at HRTs 8, 18 and 28 h. GFMB had the lowest COD concentration and produced the least average amount of hydrogen (14.2 mL) at different HRTs (Fig. 4-2 and Table 4-2). It has been reported that anaerobic fermentation increases the production of hydrogen when the reactor is abundant in OLR and carbohydrate (Kraemer and Bagley, 2005). Hydrogen production is correlated to COD removal in the presence of feedstocks. Our data shows that COD concentration was decreased from 143.8 to 56.2 g COD/L/d with apple

leading to the decrease of H_{max} from 510.8 to 349.2 mL at HRT 8h in first and second stage, respectively. Lee et al. (2010) reported that the OLR using the SL recirculation had a significant effect on the increase in H_2 content from 43.9 to 51.4% as the OLR increased from 19.5 to 58.5 g COD/L/d. The H_{max} from corn powder was less than that of apple and pear. The amylaceous carbohydrate in corn powder was more recalcitrant to biodegradation compared with starchy and saccharine carbohydrate (Josephl, 1997). The grape achieved lower hydrogen production (35.2 mL) compared with the other feedstocks. Josephl (1997) found that some pippins of fruit distribution could inhibit the fermentative reaction. In our study, soluble carbohydrate almost degraded in case of reactor containing grape waste. Plenty of pippins could be harmful for the fermentative reaction, leading to reduced hydrogen production. The lag time (λ) except for grape wastewater prior to exponential H_2 production was an average of 7-8 h. For grape wastewater λ was 3h. Shorter λ corresponds to the lower P and R_m (Table 2) (Xie et al., 2007). It should be mentioned that the length of the λ calculated from Eq. (1) is close to the values observed in the experiments as shown in Fig. 2(a), (b) and (c).

(A)



(B)



(C)

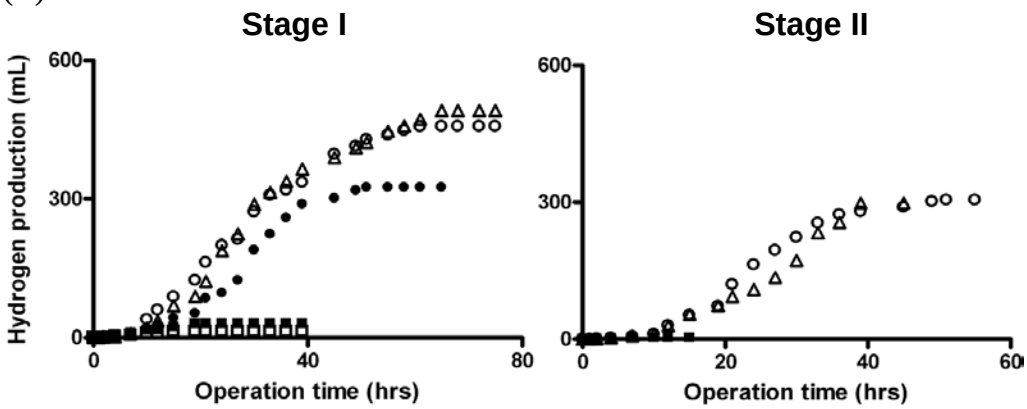


Figure 4-2. Accumulative hydrogen productions from different feedstocks at HRTs; (A) 8 h, (B) 18 h, and (C) 24 h.

Table 4-2. Summary of operational performances obtained from different feedstock

				Stage I							
HRT (h)	Feed stock	OLR (g/L/d)	Carbohydrate consumption (%)	VSS (g/L)	H_2 yield (mL/g-VS)	H_2 (%)	H_2 conversion efficiency (%)	*H (t)* (mL)	R_{max} (mL/h)	λ (h)	r^2
8	Apple	143.8	68.2	2.1 (0.1)[a]	243.4 (0.1)	56 (0.2)	11.4	510.8 (0.1)	13.9 (0.2)	7	0.99
	Pear	186.7	73.2	2.0 (0.2)	229.9 (0.2)	53 (0.3)	7.0	461.2 (0.3)	12.1 (0.3)	7	0.99
	Grape	164.2	89.3	1.9 (0.1)	18.5 (0.3)	42 (0.2)	0.17	34.8 (0.5)	2.1 (0.2)	3	0.99
	Corn powder	39.6	93.1	2.2 (0.1)	152.4 (0.1)	46 (0.1)	6.71	334.1 (0.2)	12.7 (0.4)	7	0.99
	GFBM	0.7	98	2.1 (0.2)	6.3 (0.2)	35 (0.4)	1.44	13.1 (0.1)	1.4 (0.1)	7	0.99
18	Apple	90.3	71.3	2.4 (0.3)	227.9 (0.1)	57 (0.2)	12.3	537.8 (0.1)	14 (0.2)	8	0.93
	Pear	133.2	74.1	2.2 (0.2)	222.0 (0.3)	51 (0.3)	7.4	483.8 (0.2)	12.9 (0.3)	7	0.99
	Grape	120.5	82.6	2.3 (0.1)	16.3 (0.4)	43 (0.2)	0.5	37.6 (0.1)	2.3 (0.3)	34	0.98
	Corn powder	24.3	94.9	2.2 (0.3)	179.3 (0.2)	48 (0.1)	9.7	393.7 (0.3)	13.1 (0.1)	9	0.99
	GFBM	0.3	96.2	2.3 (0.1)	6.7 (0.1)	37 (0.0)	1.9	15.5 (0.2)	1.5 (0.2)	7	0.99

HRT (h)	Feed stock	OLR (g/L/d)	Carbohydrate consumption (%)	VSS (g/L)	H_2 yield (mL/g-VS)	H_2 (%)	H_2 conversion efficiency (%)	*H (t)* (mL)	R_{max} (mL/h)	λ (h)	r^2
				Stage I							
28	Apple	41.3	72.5	2.3 (0.2)	213.6 (0.2)	47 (0.1)	14.5	490.8 (0.1)	13.8 (0.1)	8	0.98
	Pear	58.6	77.1	2.2 (0.1)	207.8 (0.3)	46 (0.2)	8.7	456.9 (0.3)	13.2 (0.3)	7	0.99
	Grape	49.7	85.3	2.3 (0.1)	13.3 (0.2)	41 (0.3)	0.5	30.9 (0.4)	2.5 (0.2)	5	0.98
	Corn powder	11.2	93.8	2.3 (0.2)	141.4 (0.1)	45 (0.2)	12.1	326.1 (0.2)	13.2 (0.1)	7	0.99
	GFBM	0.2	94.3	2.2 (0.1)	6.2 (0.1)	32 (0.2)	1.6	13.9 (0.1)	1.5 (0.1)	8	0.99

The average of pH value ranging from 5.3to 5.6 during batch reaction, [a]Numbers in parenthesis represent standard deviation.

HRT (h)	Feed stock	OLR (g/L/d)	Carbohydrate consumption (%)	VSS (g/L)	H_2 yield (mL/g-VS)	H_2 (%)	H_2 conversion efficiency (%)	*H (t)* (mL)	R_{max} (mL/h)	λ (h)	r^2
				Stage II							
8	Apple	56.2	96	2.2 (0.1)[a]	162.4 (0.2)	46 (0.2)	17.4	349.2 (0.3)	10.2 (0.1)	7	0.99
	Pear	68.5	93	2.1 (0.2)	150.7 (0.2)	46 (0.3)	14.0	315.7 (0.4)	9.4 (0.3)	7	0.99
	Grape	57.7	93	1.9 (0.1)	4.3 (0.2)	41 (0.1)	0.7	7.1 (0.1)	2.3 (0.2)	3	0.95
	Corn powder	14.6	-	2.3 (0.3)	-	-	-	-	-	-	-
	GFBM	0.2	-	2.1 (0.2)	-	-	-	-	-	-	-
18	Apple	31.5	94	2.3 (0.3)	174.9 (0.2)	49 (0.1)	24.2	402.1 (0.3)	10.5 (0.3)	8	0.98
	Pear	50.3	95	2.2 (0.2)	171.1 (0.2)	48 (0.3)	17.4	376.1 (0.2)	9.7 (0.2)	7	0.97
	Grape	46.3	92	2.2 (0.1)	4.8 (0.2)	46 (0.4)	0.7	11.6 (0.4)	2.3 (0.1)	34	0.94
	Corn powder	8.2	-	2.3 (0.1)	-	-	-	-	-	-	-
	GFBM	0.1	-	2.3 (0.2)	-	-	-	-	-	-	-

			Stage II								
HRT (h)	Feed stock	OLR (g/L/d)	Carbohydrate consumption (%)	VSS (g/L)	H_2 yield (mL/g-VS)	H_2 (%)	H_2 conversion efficiency (%)	*H (t)* (mL)	R_{max} (mL/h)	λ (h)	r^2
28	Apple	15.8	93	2.3 (0.2)	129.7	44 (0.1)	24.7	297.4(0.1)	11.1 (0.1)	8	0.96
	Pear	20.6	92	2.1 (0.2)	145.3	42 (0.2)	21.2	306.2(0.1)	10.6 (0.2)	7	0.98
	Grape	21.2	93	2.3 (0.4)	1.5	39 (0.2)	3.2	3.5(0.2)	2.1 (0.1)	5	0.95
	Corn powder	3.5	-	2.2 (0.1)	-	-	-	-	-	-	-
	GFBM	0.0	-	2.1 (0.0)	-	-	-	-	-	-	-

The average of pH value ranging from 5.3 to 5.6 during batch reaction, [a]Numbers in parenthesis represent standard deviation.

The H_{max} produced from a mixture of apple, pear and a mixture of apple, pear and grape was 449, 470 mL and 365 and 412 mL in the first and second stage, respectively (Table 3). This result shows that hydrogen production from the mixture of the RFWs was lower than the total amount of hydrogen produced from the individual RFW of apple, pear or grape. The H_{max} in the mixed RFW was generally higher in the first stage than that in the second stage fermentation. The increase of the OLR resulted in the increase of H_{max} from mixed RFW as shown in Table 3.

The average carbohydrate degradations in the first stage were 70.6, 74.8 and 85.7%, while those were 94.3, 93.3 and 92.6% in the second stage based on the organic solid content of apple, pear, and grape, respectively, at HRTs from 8 to 28 h (Table 4-2 and Fig 4-3). For the mixed RFWs, carbohydrate degradations of 71.4 and 94.2% were obtained from mixed apple and pear waste in the first and second stage, respectively, at an HRT of 18 h (Table 4-3). Corn powder carbohydrate degradation efficiency was up to 93.9 % in the first stage. In the second stage, corn powder carbohydrate degradations was undetectable due to effluent reuse as the carbohydrate had already converted into hydrogen gas in the first stage (Table 4-2). These results indicate that relatively lower hydrogen production was observed for corn powder compared to RFW. This is due to more carbohydrate conversion into hydrogen by corn powder.

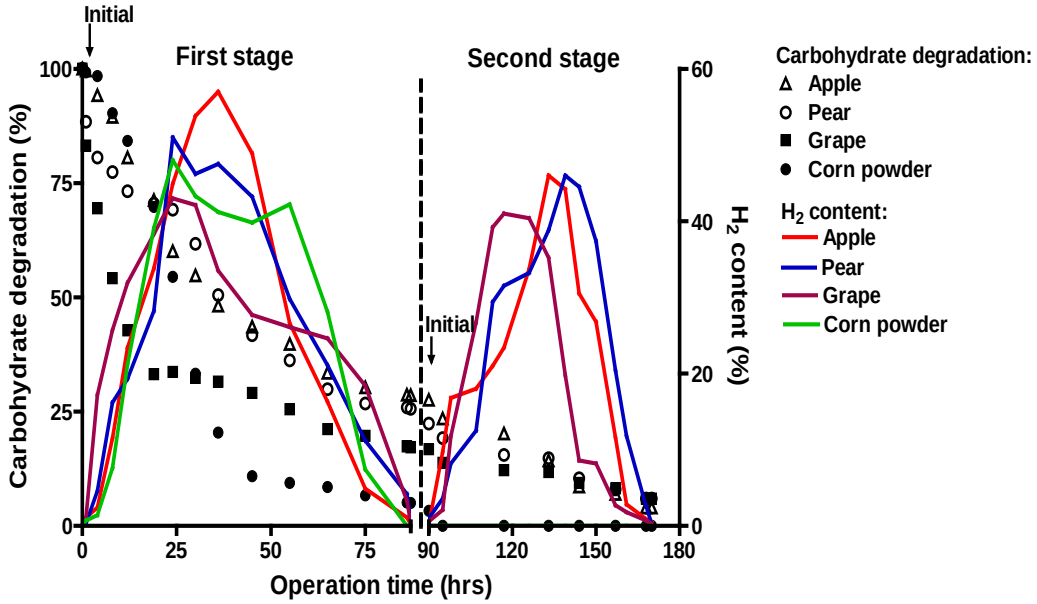


Figure 4-3. Profiles of carbohydrate degradation, H_2 content in two-stage system.

Table 4-3. Summary of operational performances obtained from two stage process for Slurry RFW at HRT 18 h

Experimental set	Feed stock	OLR (g /L/d)	pH	Carbhydrate. consumption (%)	H_{max} (mL)	VSS (g/L)	H_2 yield (mL/g-VS)	H_2 (%)	H_2 conversion efficiency (%)	R_{max} (mL/ h)	λ (h)	r^2
Stage (I)	Mixed (AP)	151.2	5.3	71.4	449.3	2.2	204.2	49	10.1	11.2	6	0.99
	Mixed (APG)	181.4	5.4	70.6	470.4	2.4	196.1	52	10.7	8.9	6	0.96
Stage (II)	Mixed (AP)	42.3	5.2	94.2	365.2	2.2	166.0	48	22.2	9.9	6	0.99
	Mixed (APG)	49.6	5.1	92.8	411.9	2.5	164.7	49	29.0	8.3	8	0.97

The RFW was composed mostly of biodegradable fractions which are readily utilizable for cell growth as well as the activation of HPB (Hwang et al., 2009). Hydrogen producing bacteria, such as *clostridium* species, are spore producers, which establish an important part of the microbial population for hydrogen production (Hwang et al., 2009). The hydrogen composition in the biogas produced from apple fermentation was about 44-54% (Table 4-2). The solution pH is an important factor in biological hydrogen production (Oh et al., 2003; Chen et al., 2008). The hydrogenic microorganisms dominated and the sulfate reducing bacteria and methanogenic bacteria could not survive at lower pH (<5.8) conditions (Hwang et al., 2009). Maximum hydrogen yields of 243.4, 227.9 and 213.6 mL/g VS were obtained from apples in the first stage at HRTs of 8, 18 and 28 h, respectively (Table 2). Hydrogen yields gradually decreased from 243.4 to 213.6, 229.9 to 207.8 and 18.5 to 13.3 mL/g VS with the decreases in OLR from 143.8, 186.7 and 164.2 to 41.3, 58.6 and 49.7 for apple, pear and grape, respectively, in first stage with an increase of HRT from 8 to 28 h. There was no difference in the average concentration of VSS (2.2 g/L) in the first and second stage at HRTs 8, 18 and 28h which has been reported by Romli (1994) and Kraemer and Bagley (2005).

Amount of hydrogen production, *H (t),* in the second stage was significantly lower than the amount produced in the first stage, which corresponded to the decreases in OLR and carbohydrate concentration (Tables 4-2 and 4-3). Fig. 4-3 shows the carbohydrate degradation along with H_2 content produced in the two

stage fermentation reactor system. The effluent discharged from the first stage was simultaneously fed into the second stage after operation was begun. Initially, at first stage from 100% carbohydrate, 42-57% of H_2 content was obtained (Fig. 3). The second stage produced 39-49% H_2 by reusing the effluents from the first stage containing 16-27% of carbohydrate. However, the H_2 production was undetectable in second stage for corn powder. This corresponds to lower carbohydrate concentration. The results showed that continuous H_2 production was achieved in the two stage using RFWs as a reuse feedstocks support, which allowed residual carbohydrate in the feedstocks.

The hydrogen production efficiency decreased with the decrease of OLR at different HRT. The maximum hydrogen production efficiency (31.8%) was obtained from apple in first stage at HRT 8h (Table 2). The presence of second stage fermentation reactor leads to increase the total hydrogen production efficiency from 31.8 to 35.8%. For pear wastewater, the H_2 conversion increased to 2.3 folds in second reactor stage with HRT 18 h. This result shows that an increase in H_2 conversion efficiency in second reactor could be due to residual carbohydrate degradation. The highest biogas production rate (R_{max}) of 14.0 mL H_2/h was obtained from the apple fermentation process at an HRT of 18 h (Table 2). The lag time prior to exponential hydrogen production was an average of 7 h with the exception of grape wastewater (4 h). Van Ginkel et al. (2005) reported R_{max} value of 10.0 mL H_2/h, which is similar to the value of apple fermentation

(14.0 mL H_2/h) but the lag phase was 35 h (ca. 5-folds longer) using apple wastewater without nutrients.

4.3.3. Volatile fatty acids concentrations

Hydrogen production is accompanied with VFAs production during anaerobic fermentation of organic substrates. The information of VFAs concentration has been used for monitoring hydrogen production (Chen et al., 2002). The major byproducts in this study were butyrate, acetate and propionate, and among them butyrate was dominant in all test runs (Fig. 4-4 and Table 4-4). The fermentation with the formation of butyric and acetic acid is generally known to be one of the most efficient ways for biohydrogen production especially by *Clostridium* sp. (Ewyernie et al., 2001). In the first stage, the average concentrations of butyric acid with rotten apples were 5438.3 ± 131.2, 6873.7 ± 182.4, and 6642.1 ± 161.7 mg/L at HRTs of 8, 18 and 28 h, respectively. The formation of butyric acid in the second stage was generally lower than the butyric acid formation in the first stage. The butyric acid is more toxic than acetic acid, but likely it is a consequence of NAD^+ regeneration (Husemann and Papoutsakis, 1997). In our study, hydrogen yield resulted in lower butyric acid concentration with high hydrogen production in second stage. This might be attributed to the consumption of butyric and acetic acid in the metabolic pathway for hydrogen production.

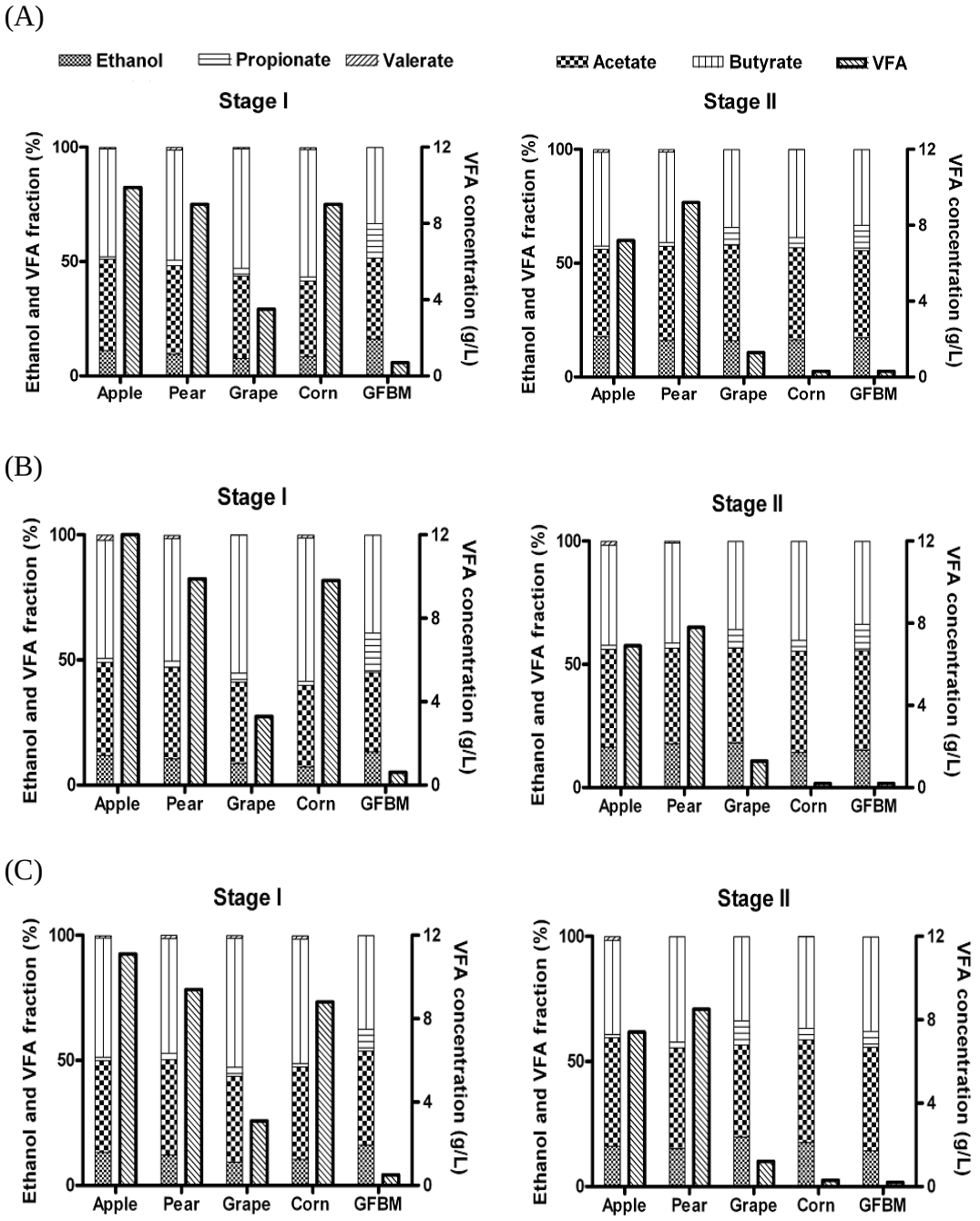


Figure 4-4. Ethanol and VFAs produced from different feedstocks at HRTs; (A) 8 h, (B) 18h, and (C) 28 h.

Table 4-4. Organic acid concentrations from stage process for Slurry RFW at HRT 18 h

Experimental set		OLR (g COD L^{-1} d^{-1})	Ethanol	Acetate	Propionate	Butyrate	Valerate	VFA (mg L^{-1})
					(%)			
Stage (I)	Mixed(AP)	151.2	12.3	36.5	1.7	48.3	1.2	9934.2(285.7)[a]
	Mixed(APG)	181.4	10.4	37.9	2.2	48.7	0.8	10860.4(277.4)
Stage (II)	Mixed (AP)	42.3	16.7	41.2	4.4	36.7	1.0	6912.8(194.7)
	Mixed APG)	49.6	15.8	45.4	6.4	32.2	0.2	7271.0(226.1)

[a]Numbers in parenthesis represent standard deviation.

In particular, the bioactivity of hydrogenesis was decreased by low carbohydrate concentration caused by the decreased OLR with corn powder. In the first stage, residual carbohydrates were almost completely consumed and converted to VFAs prior to entering the second stage of the system. The low butyric acid production in the second stage could be due to the decreased carbohydrate conversion by HPB (Fan et al., 2008). It has been reported that the production of butyrate acid decreased in the anaerobic fermentation process when hydrogen production decreased (Hawkes et al., 2002). The metabolic pathway of hydrogen fermentation with glucose degradation has been evaluated using the Butyrate/Acetate (B/A) ratio (Nandi and Sengupta, 1998) and has frequently been used as the indicator for evaluating the effectiveness of hydrogen production (Annous et al., 1996). In our study the average B/A ratios were 1.3, 1.4 and 1.4 in the first and second stage suggesting that the two stages have a similar metabolic pathway for biohydrogen fermentation. This is in agreement with a previous study which reported B/A ratios ranged from 1.1 to 1.7 (Ueno et al., 2007a). The relatively invariant B/A ratio with varying carbohydrate was presumably due to fatty acid oxidation by HPB. The B/A ratio for hydrogen production varies depending on substrates and the seed sludge (Ueno et al., 2007a).

4. Conclusions

This study demonstrated the feasibility of H_2 production from different feedstocks using a two stage system via dark fermentation The results showed that the average hydrogen production efficiency from mixed apple and grape as a RFW was 29% in the first stage and 7% in the second stage at an HRT of 18h so that the total hydrogen recovery was increased from 29% to 36% by the two stage hydrogen production system. The RFWs could be successfully used as the substrates for biohydrogen fermentation, and a highly efficient hydrogen recovery was obtained in a two stage fermentation system.

4-5. References

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CHAPTER 5

Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions

Abstract

Eukaryotic and prokaryotic microalgae are capable of hydrogen production during photosynthesis but the focus has been on photo-heterotrophic and -mixotrophic production, mostly under anaerobic and limited aerobic conditions. Here we show that three novel eukaryotic microalgae including *Chlorella vulgaris* YSL01 and YSL16 can upregulate hydrogenase expression and simultaneously produce hydrogen through photosynthesis using an inorganic carbon source under aerobic conditions with continuous illumination. We employed dissolved oxygen regimes typical of environmental conditions for eukaryotic microalgae in natural aquatic systems. Experimental data with mRNA expression and the specific activity of hydrogenase evidenced that eukaryotic microalgae enzymatically produced biohydrogen even under atmospheric conditions, which has been previously considered infeasible. The discovery of photoautotrophic H_2 production is important for assessing the ecological and algae-based photolysis implications.

Key words: Eukaryotic microalgae, Prokaryotic microalgae, Photosynthesis, Hydrogenase, Hydrogen production

5-1. Introduction

Eukaryotic microalgae generally thrive on sunlight and inorganic nutrients in freshwater environments, and are much more abundant in many natural environments than prokaryotic cyanobacteria (Gaffron and Rubin, 1942; Embley and Martin, 2006; Melis, 2009). The growth kinetic of photoautotrophic algae is dependent on the extent of available carbon sources (e.g., bicarbonate) that are mainly from dissolution of atmospheric carbon dioxide (Melis and Happe, 2001; Van de Waal, 2011). The photosynthetic process in algae results in the splitting of H_2O and resultant O_2 evolution [driven by light absorbed by photosystem II (PS II)]. Subsequently it allows transfer of electrons from photosystem I (PS I) plasto-cyanin to hydrogenase (H_2ase) through ferredoxin, which is the natural electron donor (driven by light absorbed by PS I) while forming biomass or producing energy (Chisti, 2007). Typical photosynthetic hydrogen production under strictly anaerobic conditions has been described by both indirect (PS II followed by PS I) and direct (PS II alone, i.e., PS I independent) pathways (Florin et al., 2001; Melis, 2007). This study describes the novel finding that several eukaryotic green algae can produce H_2 during inorganic carbon uptake under harsh O_2 levels that are known to inhibit hydrogenase synthesis and activity.

Review of previous reports demonstrates that photosynthetic H_2 production in both eukaryotic and prokaryotic microorganisms (e.g., *Chlamydomonas reinhardtii* and *Synechocystis* sp.) is mediated by hydrogenases under strict anaerobic conditions (Ghirardi et al., 2000; Wünschiers and Schulz, 2001; Melis,

2007), and cyanobacterial strains such as *Cyanothece* sp. are among the exception in being able to evolve H_2 even under aerobic conditions (Melies, 2009; Bothe et al., 2010), possibly due in part to dark phase respiration that rapidly consumes dissolved O_2.

H_2ases can be classified into [NiFe], [FeFe], and [Fe] based on the metal content in their active sites (Volbeda et al., 1995). It has been reported that the catalytic activity of these enzymes is strongly influenced by oxygen concentration, especially [NiFe] H_2ases are significantly inactivated in the presence of oxygen (McKinlay and Harwood, 2010). Interestingly, however, the most recent work on oxygen limitation of the activities of H_2ases in prokaryotic bacteria disclosed that [NiFe] H_2ases are tolerant to oxygen (up to 15%) in H_2 production (Allakhverdiev et al., 2010). The oxygen sensitivity of these enzymes has been regarded as one of the biggest challenges in utilizing oxygenic photosynthetic microorganisms for hydrogen production. Thus most studies on photosynthetic hydrogen production have largely relied on the experimental manipulation (e.g., intermittent illumination and/or anaerobic condition) in which dissolved oxygen was completely removed from culture media (Gaffron and Rubin, 1942; Wünschiers and Schulz, 2001).

This study describes the novel finding that eukaryotic green algal strains can produce H_2 during inorganic carbon uptake under harsh O_2 levels that are known to inhibit hydrogenase synthesis and activity. Two novel eukaryotic microalgae strains identified as *Chlorella vulgaris* YSL01 and YSL16 (Fig. 5-1 and Table 5-

1), directly produced gaseous hydrogen through photosynthesis under four different dissolved oxygen regimes using CO_2 as the sole carbon source with continuous illumination. The discovery of eukaryotic and oxygenic photoautotroph in this study that can evolve hydrogen even under aerobic conditions is possibly one of the most important findings in biohydrogen production.

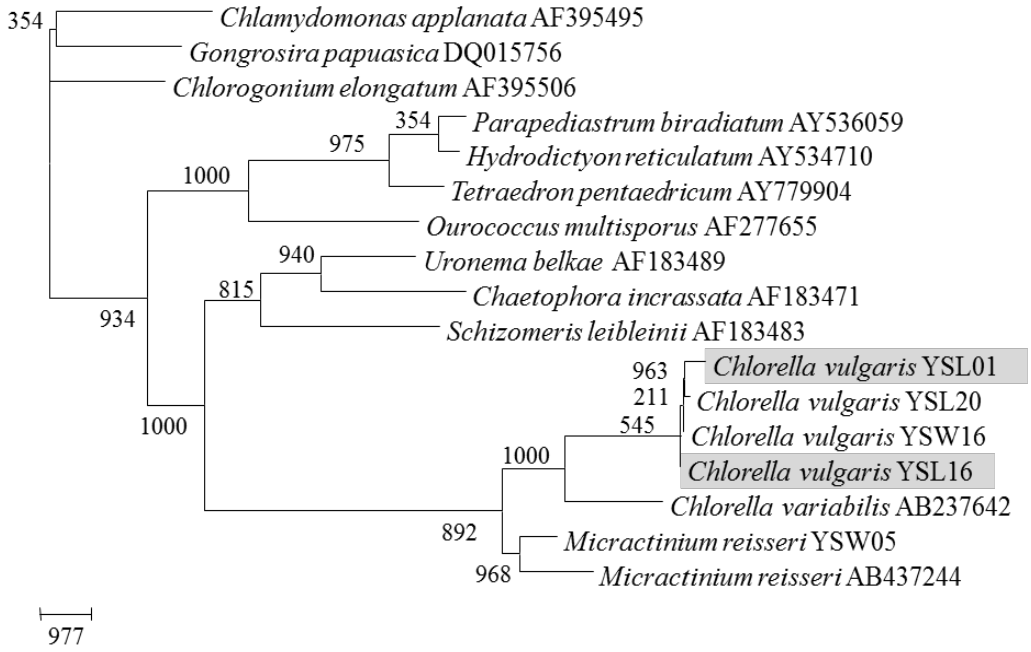


Figure 5-1. Phylogenetic tree showing the relationship among LSU rDNA D1-D2 sequences of isolates YSL01, YSL16 and YSW05, and the most similar sequences retrieved from the NCBI nucleotide database.

Table 5-1. The accession number, base pair length of the DNA fragment, and the similarity between amplified sequence and the closest relative sequence of the microalgae species isolated from lake.

Microalgae strain	Accession number	Length (nt[a])	Closest relative and GenBank accession number	Identity (%)
C. vulgaris YSL01	FR751187	883	*Chlorella vulgaris* AB237642.1	98
C. vulgaris YSL16	FR751196	873	*Chlorella vulgaris* AB237642.1	99

[a] nucleotide

5-2. Materials and Methods

5-2-1. Strain isolation and growth conditions.

Chlorella vulgaris strains (YSL01 and YSL16) were isolated from a local lake, S. Korea (Fig. 5-1). Each microalga species was inoculated into 500 mL aluminum crimp-sealed serum bottles containing 150 mL $EDTANa_2$-omitted Bold Basal Medium (BBM) and was monitored for algal growth using an optical microscope. Subcultures were prepared by inoculating 50 mL onto petri plates containing BBM (without $EDTANa_2$) solidified with 1.5% (w/v) of bacteriological agar. Further, 50 mL aliquots of the same dilution were placed into wells of a 96-well microtiter plate containing 200 mL $EDTANa_2$-omitted BBM. The experimental bottles were incubated under white fluorescent light illumination at 50 μmol/m^2/s at 27 ^oC for five weeks while shaking at 150 rpm without supplemented organic carbon source. The headspace of the serum bottles was artificially replaced with four different gas mixtures comprised of N_2, CO_2, and O_2. The initial partial pressures of both CO_2 and O_2 ranged from 5% to 15%, and atmospheric condition was also investigated. Experiments were carried out in triplicate.

5-2-2. H_2 and O_2 measurements.

H_2 and O_2 in the headspace of the serum bottles was periodically measured using a GC-14 Gas Chromatograph (Shimadzu, Japan) equipped with a thermal conductivity detector and a molecular sieve 5A 80/100 column using Ar as a carrier gas.

5-2-3. Preparation of crude cell extract and measurement of hydrogenase activity.

The crude cell extract preparation and the measurement of hydrogenase activity were performed as described by Ueno et al. (1999). Each data represents the average of triplicate measurements. After 7-8 days of cultivation, cells in the linear growth phase were harvested by centrifugation (12,000 g, 4 ^{o}C, 15 min) and washed twice with a 20 mM phosphate buffer (pH 7.5) with 1% NaCl. The resulting pellet was resuspended in an equal volume of the same buffer, and then the cell suspension was flushed with oxygen-free nitrogen gas under anaerobic conditions. After 12 h of the anaerobic adaptation process, cells were harvested by centrifugation (12,000 g, 4 ^{o}C, 20 min) and resuspended in a basal buffer containing 50 mM Tris(hydroxymethyl) aminomethane-HCl (pH 8.0), 2 mM $MgCl_2$ and 1 mM Dithiothreitol (DTT) followed by addition of powdered sodium dithionite (50 mM). The suspension was placed into a sealed steel beaker, sonicated at 300 W for 10 min under a pulsed 75%-duty-cycle condition (Sonomasher, Ulsso-Tech, Seoul, Korea), and centrifuged at 15,000 g for 20 min

at 4 ^{o}C. The crude extract was prepared under strictly anaerobic conditions at 4 ^{o}C. The hydrogenase activity was quantified by the amount of hydrogen evolved from methyl viologen which had been reduced by sodium dithionite. The hydrogen production was determined by gas chromatography with a thermal conductivity detector (GC6890A, Agilent, US). The assays were prepared in seal-lock vials (20 mL) with equal volume of liquid and gas phases. The sample (0.1-0.25 mL) was injected into 10 mL of basal buffer containing 5 mM methyl viologen and 5 mM sodium dithionite and incubated in a shaker at 28 ^{o}C for 30 min. One unit of activity is defined as the amount of hydrogenase evolving 1 μmol hydrogen gas per minute. Protein concentration in crude extracts was determined by the Bradford method using Bovine Serum Albumin (BSA) as a standard.

5-2-4. Total RNA isolation and hydrogenase mRNA expression.

Total RNA was isolated from the cells using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's instruction. The RNA concentration of each sample was determined by spectrophotometer at 260 nm. The integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, SantaClara, CA). cDNA synthesis was performed with 1 µg of total RNA in 20 µL using random primers (Invitrogen, Carlsbad, CA) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real time reverse transcriptase PCR analyses were performed using a 7500 Real-Time PCR System (Applied Biosystems Inc, Foster City, CA). Reactions were performed in a 25 µL vial containing 12.5 µL of 2X SYBR Green reaction buffer, 1 µL of cDNA (corresponding to 25 ng of reverse transcribed total RNA) and 5 pmol of each hydrogenase specific primer obtained from *Chlorella fusca* (GenBank accession no. AJ298228). After an initial incubation for 2 min at 50 ^{o}C, the cDNA was denatured at 95 ^{o}C for 10 min followed by 45 cycles of PCR (95 ^{o}C for 15 s, 60 ^{o}C for 60 s). Data analyses were performed on 7500 system SDS software version 1.3.1 (Applied Biosystems Inc, USA). All analyzed samples were normalized by the corresponding expression of 18S rRNA.

5-3. Results and discussion

5-3-1. Eukaryotic microalgae are capable of hydrogen production under aerobic conditions.

The amount of hydrogen in the headspace was monitored throughout cultivation period and the highest net hydrogen peak for each culture was illustrated (Fig. 5-2). Photoautotrophic hydrogen production by these isolates increased with increasing headspace CO_2 at 5% O_2, was unaffected at 10% O_2, and declined at 15% O_2 (Fig. 5-2). Hydrogen produced by eukaryotic algae was accumulated in the headspace of serum bottles with different cultivation conditions. The hydrogen production decreased with increasing initial O_2 in the headspace. The highest hydrogen production was achieved by *C. vulgaris* YSL01 up to 1.9 mL of H_2/L with 10% CO_2 and 5% O_2. Hydrogen was also produced even under atmospheric conditions ranging from 0.03 to 0.05 mL of H_2/L.

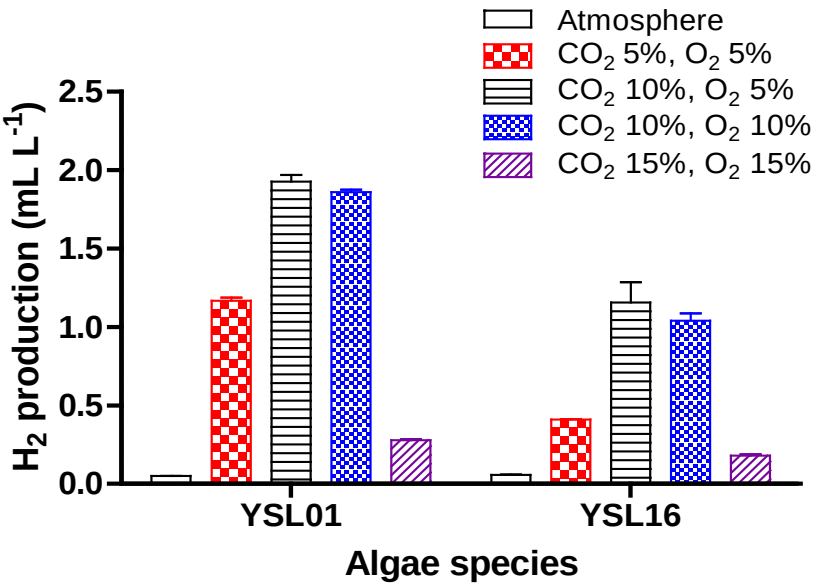
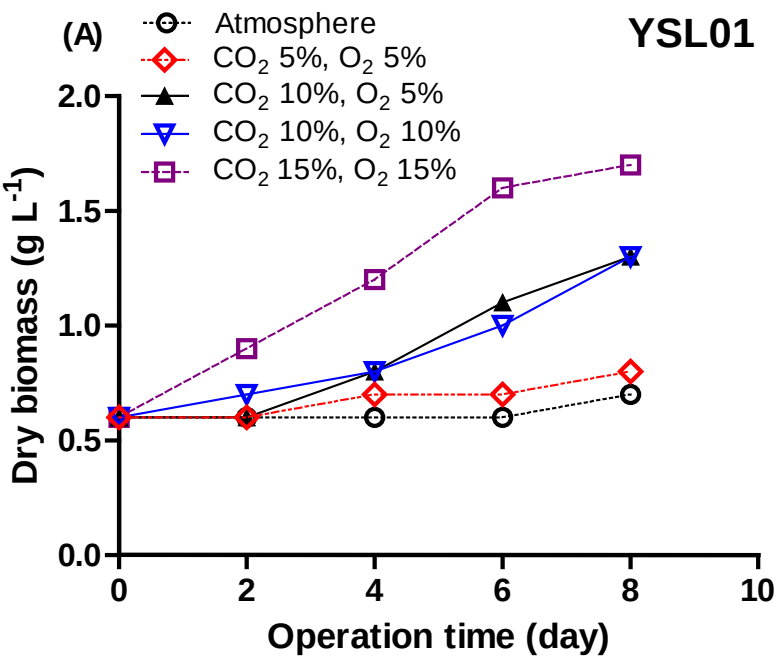


Figure 5-2. Net accumulative photoautotrophic hydrogen production by eukaryotic algae cultivated under different initial CO_2 and O_2 concentrations in the headspace.

The initial biomass concentration of *C. vulgaris* YSL01 was 0.6 g dry weight/L and varied from 0.6 to 1.7 g dry weight/L after 8 days of cultivation at the different initial CO_2 concentration ranging from 0.003 (atmosphere) to 15%. For all of the cultivation conditions the suspension pH in the serum bottles increased from 7.9 (initial) up to 8.7 (end of incubation) (Fig. 5-3A and B).

The continuous illumination for cultivation of the microalgae species resulted in relatively slower growth rates compared to previous studies employing dark/light cycles (Melis and Happe, 2001; Oncel and Sukan, 2011). The average growth rates under continuous illumination were mainly dependent upon CO_2, while little dependence on O_2 was observed.



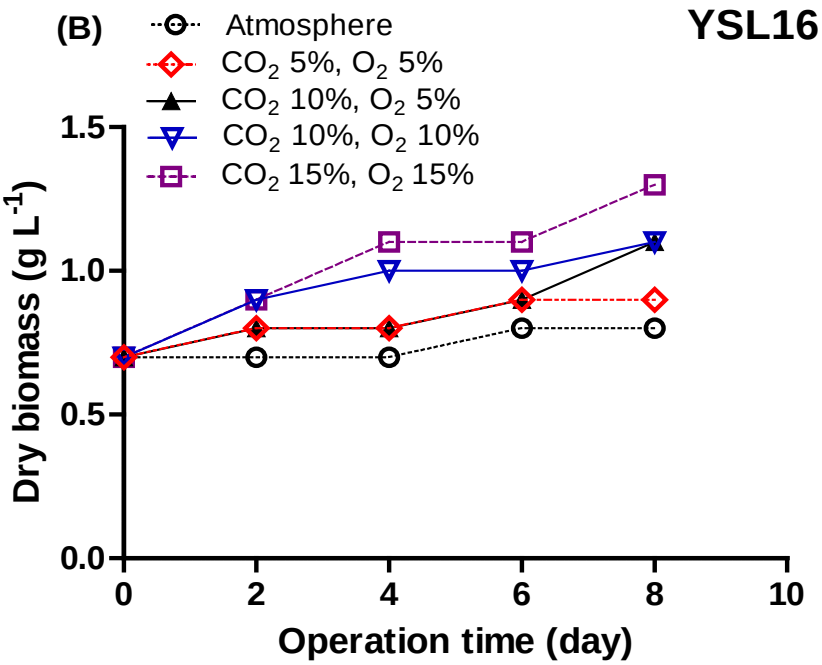


Figure 5-3. Variation in the dry cell weight of eukaryotic algae cultivated under different initial CO_2 and O_2 in the headspace (B) *Chlorella vulgaris* YSL01 and (C) *Chlorella vulgaris* YSL16

Photosynthesis in these photoautotrophic green algae directly evolved gaseous hydrogen under aerobic conditions. The photoautotrophic hydrogen production was greatly enhanced when supplemented with high concentrations of CO_2, coinciding with the previously reported work in which the cell density of photoheterotrophs growing in culture media governed the photoheterotrophic hydrogen production under anaerobic conditions (Bala and Murugesan, 2011; Oncel and Sukan, 2011). The cumulative hydrogen production increased as the headspace CO_2 concentration was increased from 0.003% (atmosphere) to 10% at

a low O_2 concentration (5%), and remained nearly constant when headspace O_2 was increased from 5% to 10% at a given concentration of CO_2 for all microalgae species. Elevating the O_2 level up to 15% resulted in a dramatic decrease in hydrogen production even when using high inorganic carbon (15% CO_2) growth medium, in which the rate of algal growth was much higher than the cell growth achieved by the other culturing conditions with the lower CO_2 concentrations (from 0.003 to 10%) (Fig. 5-3A). These results indicate that generation of hydrogen by the oxygen-evolving photoautotrophic algae was strongly correlated with oxygen level in the growth medium, which was attributed to the oxygen sensitivity of hydrogenase. A slow rate of H_2 production by photosynthetic microorganisms due to oxygen sensitivity of hydrogenase was reported previously (Bala and Murugesan, 2011; Oncel and Sukan, 2011), and thus the sensitivity of hydrogenase genes to oxygen has been implicated as a major obstacle to improve the phototrophic biological hydrogen production (Lubitz et al., 2008).

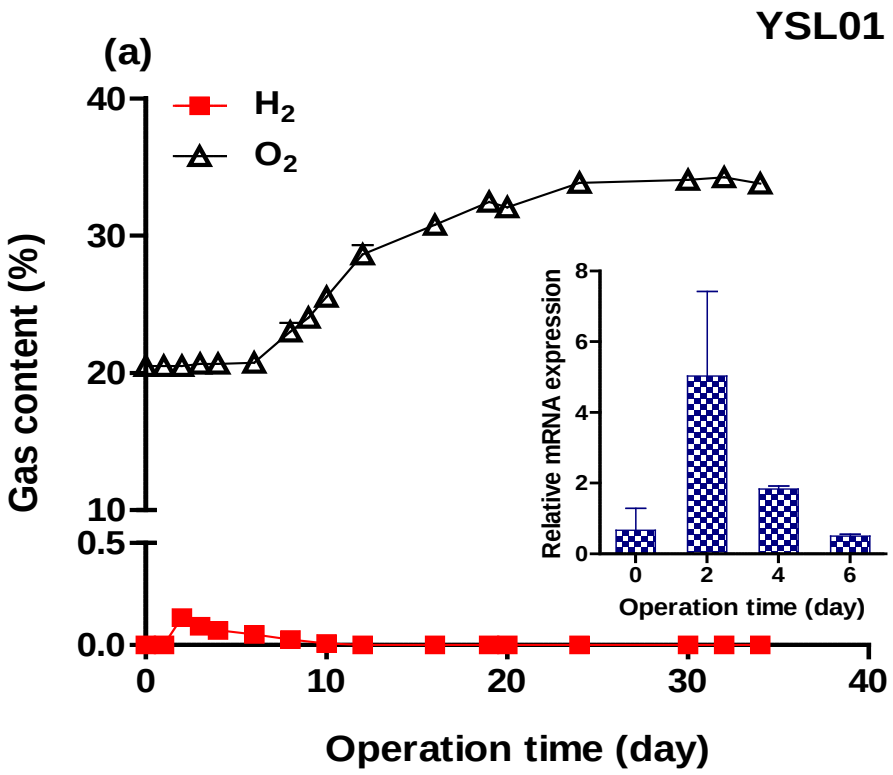
Our study demonstrates that strains of the eukaryotic microalgae *Chlorella vulgaris* is capable of hydrogen production under aerobic conditions. Chader et al (2009) showed that *Chlorella* sp. (an eukaryotic microalgae) was able to produce less amount of hydrogen with an O_2 partial pressure of up to 15% in the headspace under mixtrophic conditions. Throughout 8 days of cultivation, the highest hydrogen production was achieved by *C. vulgaris* YSL01 supplemented with CO_2 under aerobic conditions (with continuous illumination). A relatively small but noticeable amount of hydrogen production was observed even with atmospheric

conditions, suggesting that a different amount of H_2 can be produced throughout the different oxygen regimes encountered as a function of depth in natural waters. This finding of photoautotrophic hydrogen production by eukaryotic algae under different oxygen levels (including atmospheric condition) is ecologically important where the massive growth of algae can change the local environmental conditions in terms of H_2 bioavailability.

5-3-2. Hydrogen production is mediated by hydrogenease at a high oxygen concentration.

Hydrogenase mRNA, measured by real-time Reverse Transcription (RT) PCR, was synthesized at high concentrations of oxygen (≥21%, initially atmospheric condition) and its relative concentration correlated with photosynthetic hydrogen production (Fig. 5-4). The kinetics of hydrogen production by two different microalgae isolates were monitored for up to 34 days under oxygenic, CO_2-limited (closed system), and photoautotrophic conditions using crimp-sealed serum bottles. During the period of hydrogen production, normalized expression of *hydA* level was quantitatively determined. Conversely, the decrease of hydrogen production was correlated with decreasing hydrogenase mRNA levels, especially for *C. vulgaris* sp.. This result confirms the oxygen tolerance of either hydrogenase or hydrogenosome in the algal cell exposed to high oxygen concentration for a long period of time. Headspace hydrogen reached a maximum concentration of 0.15-0.69% before 10 days of cultivation regardless of the algae species examined, which was significantly influenced by the amount of oxygen. Hydrogen was also produced even under atmospheric conditions ranging from 0.03 to 0.05 mL of H_2/L (Figs. 5-2 and 5-4). The produced hydrogen was decreased with increasing oxygen content in the headspace, and was completely removed within 7 days after the peak of hydrogen production. We found no further hydrogen production at very high partial pressures of oxygen (e.g., O_2 ≥35% for *C. vulgaris* YSL16). An analogous observation was shown in an earlier work in which hydrogen was

consumed by photoheterotrophic eukaryotes under the dark/light anaerobic conditions (Gaffron and Rubin, 1942; Melis and Happe, 2001).



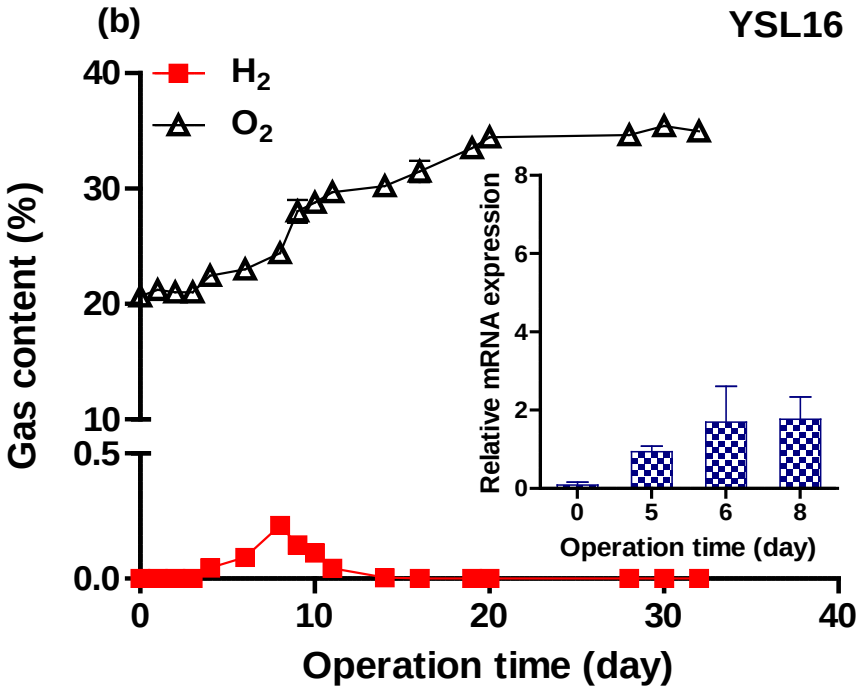


Figure 5-4. Photosynthetic hydrogen production and oxygen evolution by two different eukaryotic algae strains and the identification of their hydrogenase mRNA synthesized during oxygenic and photoautotrophic cultivation of the eukaryotes under atmospheric conditions: (a) *Chlorella vulgaris* YSL01 and (b) *Chlorella vulgaris* YSL16.

5-3-3. Specific hydrogenase activity is correlated with initial O_2 levels.

The specific activity of hydrogenase in these microalgae isolates cultivated under various headspace oxygen levels was determined after replenishing with oxygen-free nitrogen for 24 h in order to fully activate hydrogenase in the algae cells under strictly anaerobic conditions. The specific activity of hydrogenase decreased with increasing initial O_2 in the headspace for all investigated microalgae species (*C. vulgaris* YSL01 and YSL16), indicating that the enzyme activity was strongly influenced by the amount of oxygen levels (Figs. 5-2 and 5-5). The intrinsic hydrogenase activity was relatively higher for both cumulative hydrogen production on the lower O_2 conditions (5-10%) compared to the hydrogen production at the higher O_2 enriched environment (atmosphere), but there was a weak correlation between microalgae species growth and specific intrinsic hydrogenase activity (Figs. 5-3 and 5-5) due to the fixed inorganic carbon concentration.

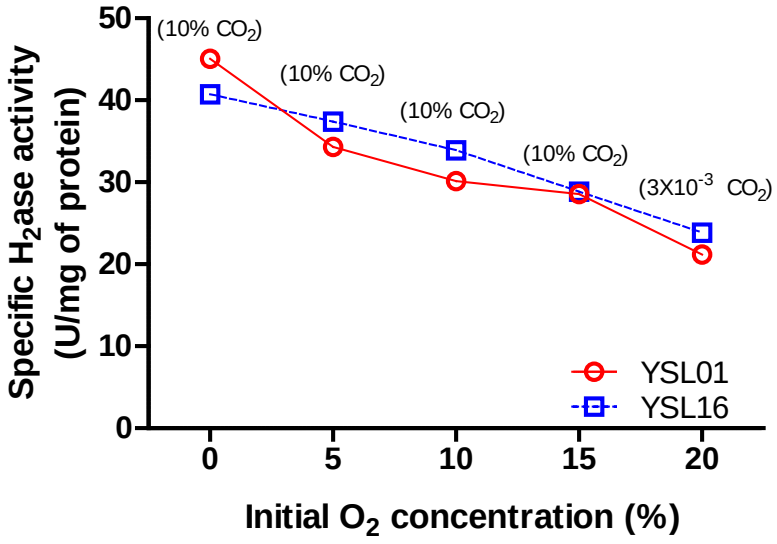
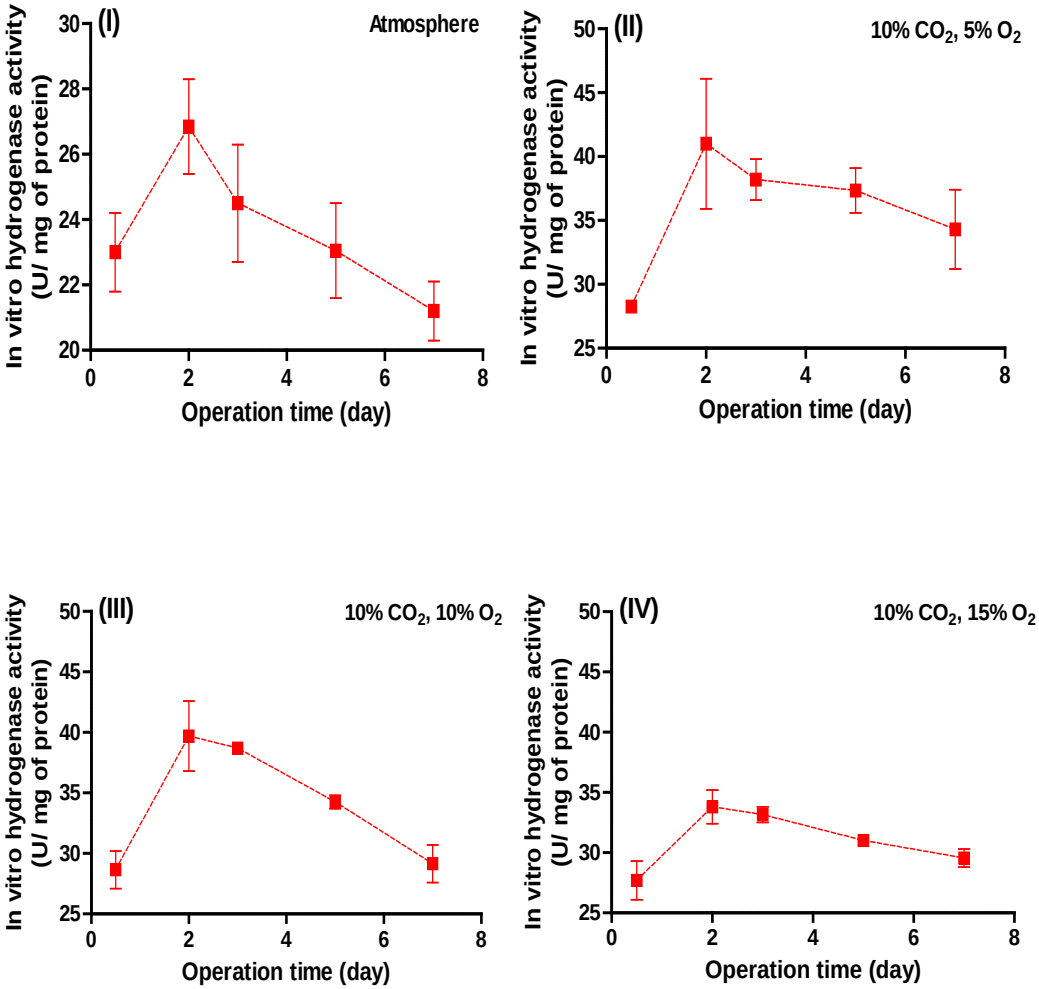


Figure 5-5. Specific activity of hydrogenase in microalgae as a function of the initial O_2 concentration in the headspace. The parentheses represent the initial CO_2 concentration. Individual data points represent the average of two independent experiments.


(I)
Atmosphere
In vitro hydrogenase activity (U/ mg of protein)
Operation time (day)
(II)
10% CO2, 5% O2
In vitro hydrogenase activity (U/ mg of protein)
Operation time (day)
(III)
10% CO2, 10% O2
In vitro hydrogenase activity (U/ mg of protein)
Operation time (day)
(IV)
10% CO2, 15% O2
In vitro hydrogenase activity (U/ mg of protein)
Operation time (day)

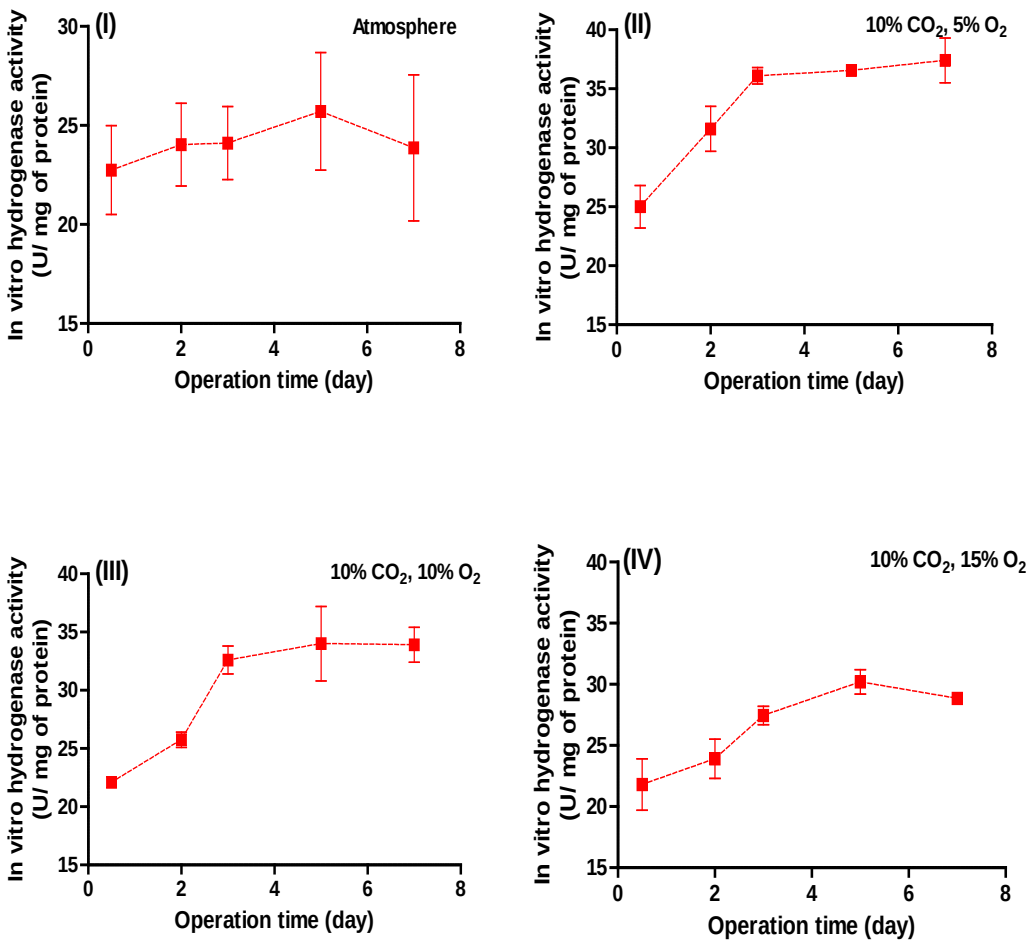


Figure 5-6. Oxygen sensitivity of *in vitro* hydrogenase in microalgae cultivated under different initial O_2 in the headspace: (a) *Chlorella vulgaris* YSL01 and (b) *Chlorella vulgaris* YSL16.

In vitro methyl viologen mediated hydrogenase activity for each of different O_2 levels at 10% CO_2 was showed in Fig 5-6. Hydrogenase is extremely sensitive to oxygen, and their activity is inhibited irreversibly, even upon exposure to low concentrations of oxygen. Despite the highest specific hydrogenase activity and cell growth rate of the microalgae species, the poor hydrogen productivity observed at a high initial partial pressure of oxygen suggests that (i) the hydrogenase activity could be impeded by high oxygen concentrations ≥15% and/or (ii) the hydrogen consumption rate by microalgae biomass would be accelerated to a much greater extent than the photosynthetic hydrogen production at a certain partial pressure of oxygen.

5-4. Conclusions

We have demonstrated that the newly isolated eukaryotic microalgae from different environmental niches are capable of photoautotrophic hydrogen production even under aerobic conditions with a continuous illumination. The measurements of both relative mRNA and specific activity of hydrogenase revealed that hydrogen production was facilitated by hydrogenase synthesis even when an atmospheric level of oxygen existed. This result provides an evidence of naturally evolved oxygen-tolerant hydrogenase in eukaryotic algae, thus further study may open a new avenue of continuous biophotolysis for hydrogen production and an opportunity to apply artificial hydrogenase-based biomimetic photovoltaic cell using eukaryotic algae.

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CHAPTER 6

Photo-heterotrophic hydrogen production by a microalga from acetate- and butyrate- enriched wastewater

Abstract

This study was conducted to evaluate the feasibility of using a microalga *Micractinium reisseri* YSW05 (*M. reisseri* YSW05) for hydrogen production with photo-heterotrophic cultivation for which the microalga requires light when using organic compounds as a carbon source. Three different culture media as a function of solution pH were prepared using anaerobic digestion effluent in which acetate and butyrate (Volatile fatty acids; VFAs) ranged from 1400 to 1550 mg/L and from 840 to 880 mg/L. The microalga species was cultured on the fatty acids-enriched bioeffluent under aerobic conditions without CO_2 for 23 days at an initial pH of 4.9, 6.8, or 8.0. Uptake of either acetate or butyrate by *M. reisseri* YSW05 was negligible at pH 4.9, while increasing the initial solution pH resulted in significant reduction of residual fatty acids in the aqueous solution; especially more than 90% of acetate was assimilated by the microalga after 15 days of cultivation at pH 6.8 or higher. The pH dependence of both microalgal growth rate and subsequent hydrogen production was also disclosed. The specific activity of microalgal hydrogenase was increased with increasing the solution pH from 4.9 to 8.0, which coincided with the highest hydrogen production (31 μmol/mg Chl

a/h) achieved by the photo-heterotrophic growth of *M. reisseri* YSW05 at pH 8.0. These results demonstrate that a mixture of acetate and butyrate in the anaerobic digestion effluent that is commonly produced by the conventional microbial fermentation of biosolid wastes from municipal wastewater treatment facilities can be an effective substrate for aerobic photo-heterotrophic cultivation of microalgae and subsequent bioenergy production.

Key words: Photo-heterotrophic H_2 production; Microalgae; Anaerobic wastewater effluent; Butyrate; Acetate

6-1. Introduction

There is an increasing interest in hydrogen production from renewable resources (Levin et al., 2004), and biological hydrogen production is one of the promising approaches through either photosynthesis (Esper et al., 2006; Ghirardi et al., 2007) or fermentation (Hwang et al., 2011) pathway that is inspired by enzymes (Tard et al., 2005; Cheah et al., 2007). Several photosynthetic microalgal and bacterial species possessing nitrogenase and/or hydrogenase enzymes have been studied as prospective model organisms for photobiological hydrogen production (Tamagnini et al., 2007). Biological methods for hydrogen production are preferable due to the advantages of using low-cost feedstock such as sunlight, carbon dioxide, and organic wastes as substrates for environmentally benign conversions under moderate conditions (Benemann et al., 1973). The acidogenic bacteria can convert various complicated organic substrates into hydrogen and volatile fatty acids (VFAs), which in turn will inhibit the fermentative hydrogen production process (Basak and Das, 2007). However, the effluent VFAs from the acidogenic process can be further decomposed into hydrogen by phototrophic bacteria shown by Chen et al. (2008) with ca. 52% hydrogen yield. Butyrate and acetate are the major fractions in VFA components derived from acidogenic hydrogen production process. Thus, to develop an applicable technology for hydrogen production, further efforts are needed to decompose the anaerobic fermentation effluent (acetate/butyrate) and subsequently convert them to hydrogen. The VFA can be stimulatory, inhibitory, or even toxic to the

fermentative bacteria depending on their concentrations (Stewart, 1975). Similarly, a low concentration of butyrate may have no impact on hydrogen production by photosynthetic microorganisms, while high butyrate concentration can lead to a strong inhibition on the photo-hydrogen production.

The photosynthetic bacteria (e.g., *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris*) and phototrophic bacteria have been studied to produce hydrogen using commercial grade pure butyrate and/or acetate as the substrate (Lee et al., 2007). Soluble metabolites can be utilized by photosynthetic bacteria to produce additional hydrogen. Although it has been recognized that the efficiency of hydrogen production is substantially affected by solution pH as well as acetate/butyrate concentration, no attempts have been made on the effects of different pH conditions on the performance of photo-heterotrophic hydrogen production system using acetate- and butyrate- enriched wastewater effluent. In order to evaluate the optimal the potential photo-heterotrophic hydrogen production from acetate and butyrate- enriched wastewater, it would be beneficial to have a good understanding on microalgal activity during the hydrogen production for optimizing substrate utilization, hydrogen production rate, and hydrogen yield in the photo-heterotrophic hydrogen production system.

The main objective of this research was to quantitatively determine the feasibility of using fatty acids derived from the conventional fermentation of biosolid wastes as a primary substrate for photo-heterotrophic cultivation of the microalga *Micractinium reisseri* YSW05 (*M. reisseri* YSW05). This research

intended to evaluate the impact of initial medium pH on the rate of microalgal growth and to maximize hydrogen production by the microalga under aerobic conditions. This work is distinguished from other investigations in the following ways: (1) we demonstrated hydrogen production during the photo-heterotrophic growth of *M. reisseri* YSW05 under aerobic conditions without CO_2 at a slightly alkaline or neutral pH; (2) the fatty acids as a byproduct of the conventional microbial fermentation were employed as a feedstock for microalgal cultivation, which might reduce the operating expenses as well as minimize the impact on the environments; and (3) we quantitatively determined the uptake of the fatty acids mainly consisting of acetate and butyrate and also demonstrated that the conversion rate of the fatty acids by *M. reisseri* YSW05.

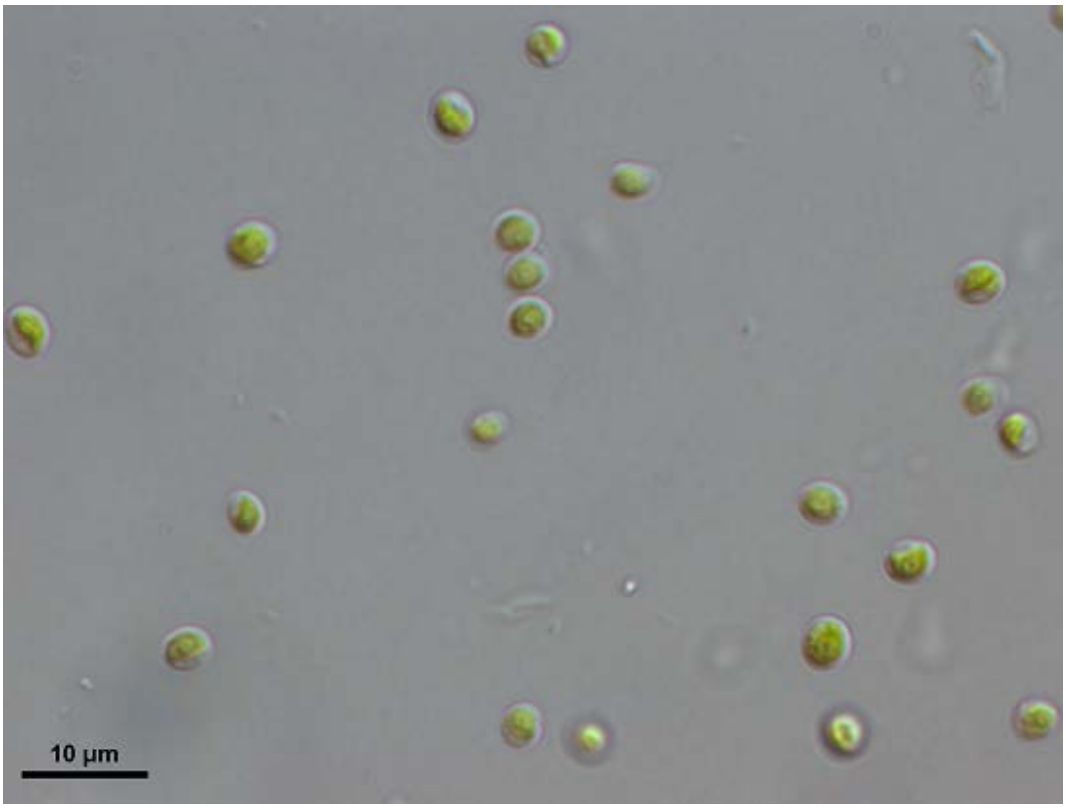
6-2. Materials and Methods

6-2-1. Isolation, purification, and identification of microalga

Microalgal was isolated from the effluent of a municipal wastewater treatment plant (Wonju Water Supply and Drainage Center, South Korea) (Abou-Shanab et al., 2011). The microalgae was cultured in 50% Bold Basal Medium (BBM) (1:1 v/v BBM and filter sterilized wastewater effluent) (Bischoff and Bold, 1963). A solution of 10 mL of *Micractinium reisseri* YSW05 suspended culture (OD 680 nm = 1.4 and VSS = 1.9 g/L) was inoculated in 100 mL of autoclaved BBM medium in 250 mL flasks. The inoculated culture was incubated on a rotary shaker (SH-804, Seyoung Scientific) at 27 ^{o}C and 150 rpm under continuous illumination

using a white fluorescent light at intensities of 40/μm^2/s for three weeks. Green algae biomass doubles their biomass within 13 to 17 days during the exponential growth phase. The morphology of *M. reisseri* YSW05 observed under a light microscope (Fig. 6-1 A).

(A)



(B)

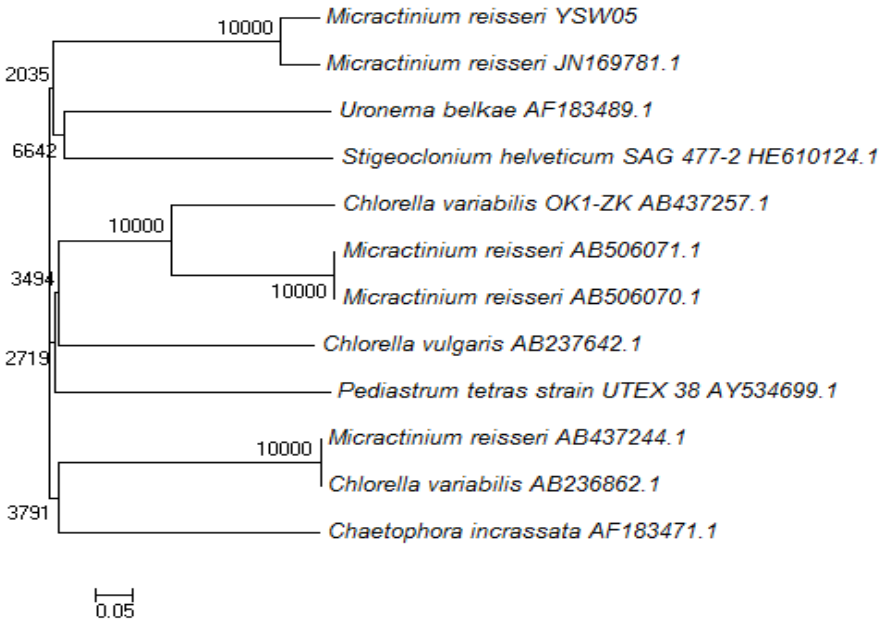


Figure 6-1. Cell morphology of *Micractinium reisseri* YSW05 observed under (A) a light microscope (x1500) at pH 8.0, and (B) phylogenetic tree showing the relationship between the LSU rDNA D1-D2 sequence of *Micractinium reisseri* YSW05 and the most similar sequences retrieved from the GenBank database.

6-2-2. PCR amplification and phylogenetic analysis

PCR amplification of the genomic DNA isolated from microalga with the universal forward and reverse primers was conducted. A single band of amplified LSU rDNA (D1-D2) product with a size of ~ 850-bp was recorded. The LSU-rRNA gene has a higher evolutionary rate compared with the SSU-rRNA gene (Sonnenberg et al., 2007) and should offer a better tool for the discrimination of closely related species using short diagnostic sequences. The DNA sequence was published in the NCBI databases under the specific accession number (FR751195). The phylogenetic tree constructed using MEGA 4.0 for the isolated strain (*M. reisseri* YSW05) was obtained from the LSU-rDNA D1-D2 sequence (Fig. 6-1 B). The LSU-rDNA sequence analysis showed that the isolated YSW05 can be ascribed to the *M. reisseri* JN169781 with a similarity of 93% (Table 6-1).

Table 6-1. The accession number, base pair length of the DNA fragment, and the similarity between amplified sequence and the closest relative sequence of the microalgae isolated from a wastewater treatment plant

Microalgae strain	Accession number	Length (nt[a])	GenBank accession number	Similarity (%)
Micractinium reisseri YSW05	FR751195	880	*Micractinium reisseri* JN169781	93

[a] nucleotide

6-2-3. Anaerobic digestion effluent and experimental batch setup

Sludge used in this study was collected from the anaerobic digesters of a municipal wastewater treatment plant (Water Supply and Drainage Center, South Korea). The anaerobic wastewater was acclimatized to a wastewater (COD 15 g/L) for 1 month in an anaerobic chemostat reactor. Synthetic medium and the acclimated sludge was mixed in 1:1 ratio (v/v) and added to a 4-L reactor, which was operated in chemostat mode at 35 ^{o}C with hydraulic retention time (HRT) of 12 h for one month (Hwang et al., 2011). Solution pH, COD, and volatile fatty acids (VFA) were 4.9, 2960 and 2430 mg/L in the anaerobic wastewater effluent.

Batch experiments were conducted at 25 ^{o}C in 120 mL serum vials. Each batch experiment was conducted in duplicate. The effects of initial pH (ranging 4.9-8.0) on hydrogen production from organic carbon enriched anaerobic wastewater effluent were investigated for 23 d of incubation period. The wastewater was abundant in acetate (1400-1550 mg/L) and butyrate (840-880 mg/L) which caused different state of algae growth for phototrophic H_2 production under continuous illumination using a white fluorescent light at intensity of 40/μm^2/s. The headspace of each bottle was flushed with N_2 and O_2 (85:15 v/v) gas mixture and sealed tightly with a butyl rubber stopper and an aluminum crimp.

6-2-4. Total RNA isolation and gene expression analysis

H_2ase activity was quantified by the amount of hydrogen evolved from methyl viologen (MV) which had been reduced by sodium dithionite. The hydrogen formation was determined by gas chromatography (GC6890A, Agilent, US). The assays were performed in seal-lock vials (20 mL) with equal volumes of the liquid and gas phase. The sample (0.1-0.25 mL) was injected into 10 mL of basal buffer containing 5 mM MV and 5 mM sodium dithionite and incubated in a shaker at 28 °C for 30 min. One unit of activity is defined as the amount of H_2ase evolving 1 μmol hydrogen gas per minute. Protein concentration in crude extracts was determined by the Bradford method (Bradford, 1976) using Bovine Serum Albumin (BSA).

Total RNA was isolated from the tissues using the QIAgen RNeasy kit (Qiagen, Valencia, CA), according to the manufacture's instruction. The RNA concentration of each sample was determined by spectrophotometer at 260nm. The integrity of each RNA sample was evaluated using the Agilent 2100 BioAnalyzer (Agilent Technologies, SantaClara, CA). cDNA synthesis was performed with 1μL of total RNA in 20 μL using random primers (Invitrogen, Carlsbad, CA) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Real time quantitative PCR analyses for the genes were performed using the 7500 Real-Time PCR System (Applied Biosystem, Foster City, CA). Reactions were performed in a 25 μL volume containing 12.5 μL of 2X SYBR Green reaction buffer, 1 μL of cDNA (corresponding to 25 ng of reverse transcribed total RNA) and 5pmol of each

primer. After an initial incubation for 2 min at 50 °C, the cDNA was denatured at 95 °C for 10 min followed by 45 cycles of PCR (95 °C, 15 s, 60 °C, 60s). Data analyses were performed on 7500 System SDS software version 1.3.1 (Applied Biosystem). All the samples were normalized by the corresponding expression of 28S rRNA.

6-2-5. Analytical methods

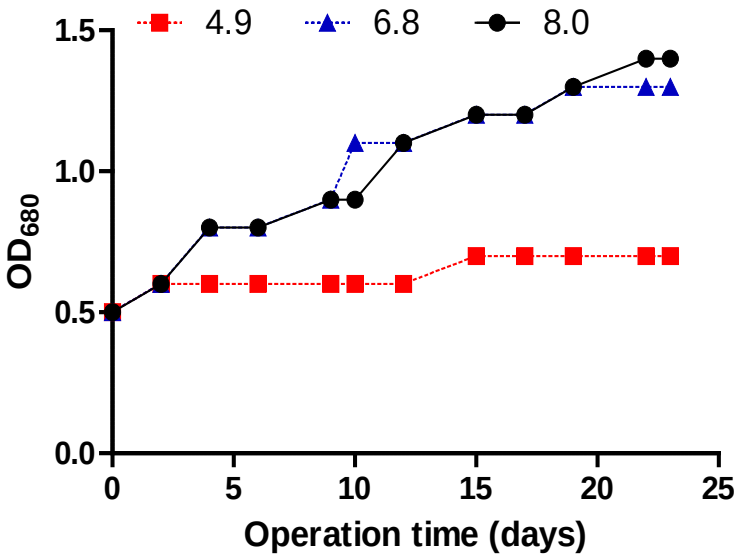
The solution pH was measured with a pH meter (Thermo Orion 290A, Orion Corporation), and H_2 gas in the vessel headspaces was periodically measured using a gas chromatograph (Shimadzu GC-14, Japan) equipped with a thermal conductivity detector and a molecular sieve 5A (80/100 6 ft×1/8 ft) column using Ar as a carrier gas. The temperatures of the injector, the detector and the column were maintained at 80, 110 and 60 °C, respectively. VFA were analyzed using a flame ionization detector (ShimadzuGC-8A, Japan) equipped with a glass column packed with 10% REOPLEX 400. The temperatures of injector port, detector and column were 240, 240, and 140 °C, respectively. Total Chl *a* and *b* was spectrophotometrically determined by extraction in 95% ethanol (Harris, 1989). Total chlorophyll was selected as the standard for comparison to correlate hydrogenase activity with the light-absorbing capacity of the cultures. Under the given culturing conditions, total chlorophyll per cell was similar amongst the strains examined.

6-3. Results and discussion

6-3-1. Effect of solution pH on the microalgal growth and fatty acid consumption

The results of batch experiments with anaerobic digestion effluent at different pH conditions are shown in Fig. 6-2 in which the optical density and dry cell weight concentration are plotted over time.

(A)



(B)

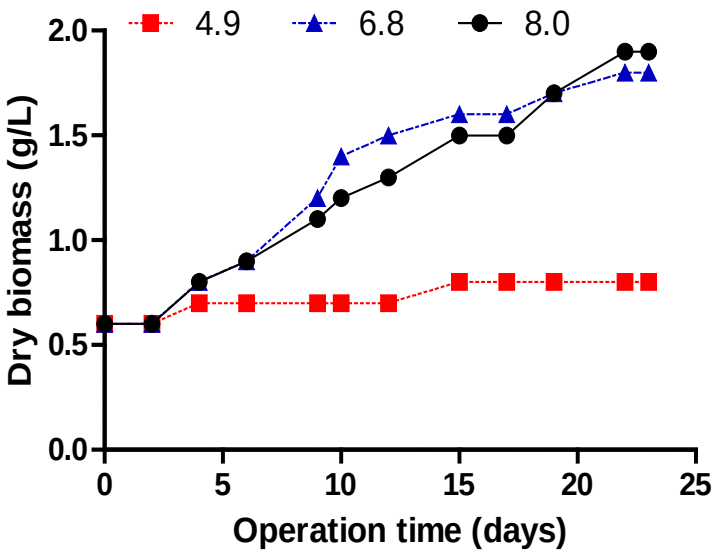


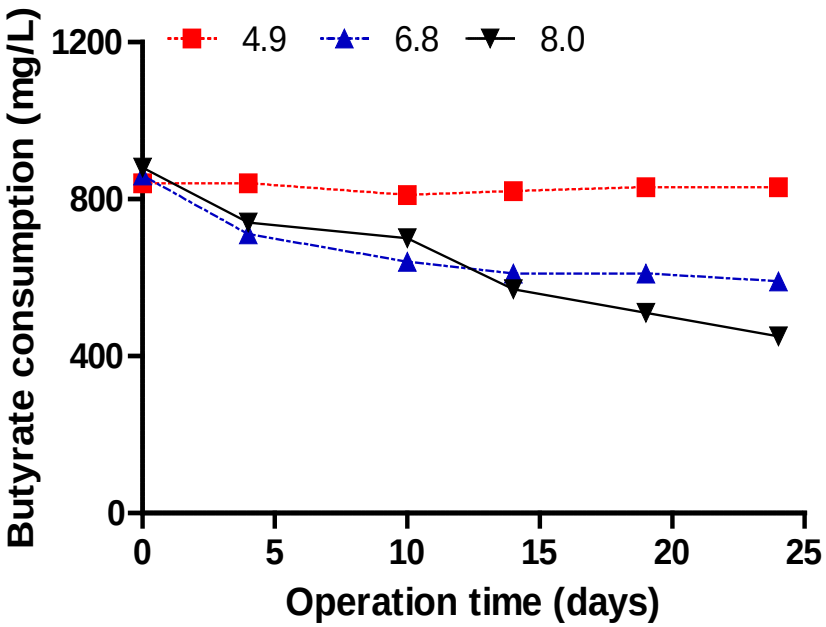
Figure 6-2. Effect of initial solution pH on the growth rate of *M. reisseri* YSW05: (a) optical density at 680 nm and (b) dry cell weight concentration vs. cultivation time.

The average growth rates of 0.7, 1.7 and 1.9 g dry weight/L were observed for photo-heterotrophic cultivation of *M. reisseri* YSW05 at pH 4.9, 6.8, and 8.0, respectively. The microalgal growth was nearly zero at pH 4.9, reflecting the slow progress of photosynthesis via uptake of organic carbon sources. Increasing the solution pH to 6.8 or higher resulted in improved growth rate of *M. reisseri* YSW05. A linear growth of microalga was observed throughout the 23 day

cultivation period at pH 8.0, while for the first 10 days of cultivation at pH 6.8 the rate of microalgal growth was much higher than achieved by the other cultures (e.g. showed 48 h of doubling time at the rapid growth phase). These trends were almost identical to those found in the result of the OD_{680} measurements. Microalgae grow rapidly and usually double their biomass within 24 h during the exponential growth phase in synthetic media (Chisti, 2007).

Fig. 6-3 shows the uptake of fatty acids by *M. reisseri* YSW05 as a function of solution pH. Neither acetate nor butyrate was biodegraded at pH 4.9, coincided with the result showing negligible growth at the same pH. On contrary, increasing the solution pH to 6.8 or higher resulted in significant removal of the fatty acids from anaerobic digestion effluent.

(A)



(B)

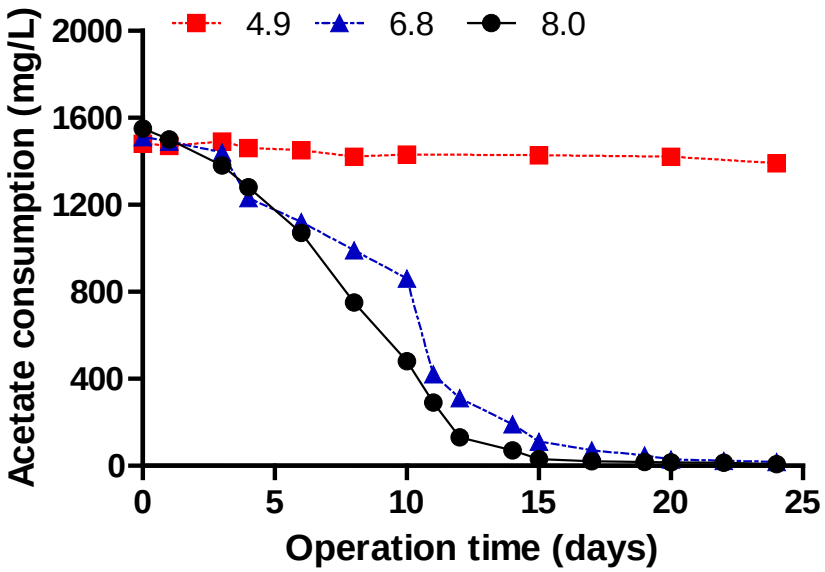


Figure 6-3. Biodegradation of (a) butyrate and (b) acetate by *M. reisseri* YSW05 under the aerobic conditions at an initial solution pH of 4.9, 6.8, or 8.0.

M. reisseri YSW05 could degrade butyrate, but acetate was preferentially assimilated by the micoalga. More than 90% of acetate was assimilated by the microalga after the 14-15 days cultivation, while 51% of initial butyrate remained even at the end of cultivation for 23 days at pH 6.8 or higher.

6-3-2. Effect of solution pH on hydrogen production and H_2ase activity

Two reaction pathways for microalgal hydrogen production form acetate/butyrate could be involved including 1) the immediate hydrogen production from both acetate and butyrate, and 2) two-step hydrogen generation from butyrate which was biotically converted to acetate follow by immediate hydrogen production. Up to 10 moles of hydrogen can be theoretically produced per each mole of butyrate, while earlier reports show that the hydrogen yield ranged from 8.4to 12.9 % (Barbosa et al., 2001; Lee et al., 2007). Occasionally, butyrate in the mixed substrate with acetate mainly contributes cell growth and greater conversion rate of acetate to hydrogen. It has been reported that a blue-green alga *Rhodopseudomonas Faecalis* (RLD-53) grew with mixed substrate (acetate and butyrate) was not able to convert butyrate to H_2. Instead, the presence of high concentration of butyrate (2200 mg/L) promoted the conversion of acetate (1500 mg/L) to hydrogen (Ren et al., 2008). Cumulative hydrogen production from anaerobic wastewater effluent at different pH conditions was estimated using the modified Gompertz equation (Lay et al., 1997), which illustrated that hydrogen production increased as the solution pH was increased from 4.9 to 8.0 (Fig. 6-4).

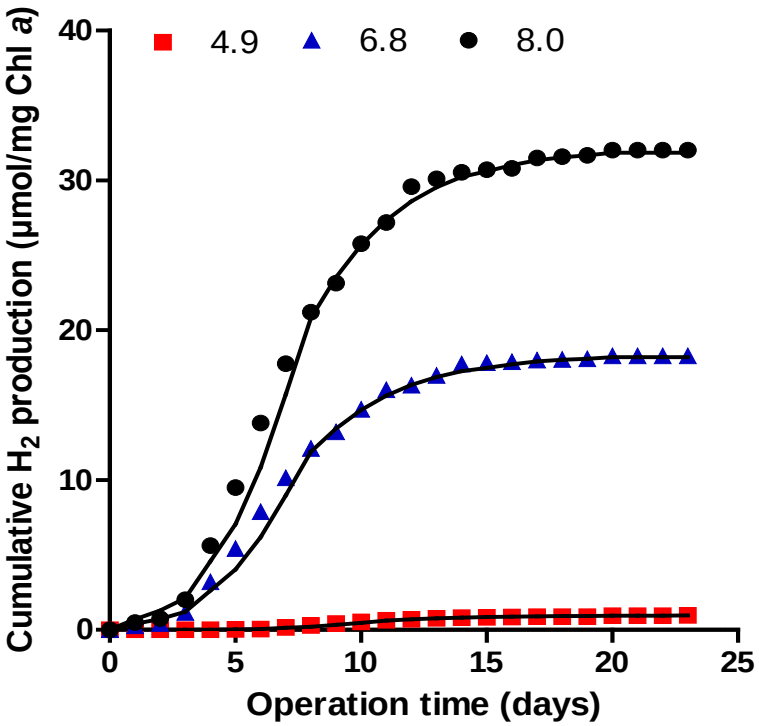


Figure 6-4. Cumulative hydrogen production during the photo-heterotrophic growth of *M. reisseri* YSW05 under the aerobic conditions without CO_2 at an initial solution pH of 4.9, 6.8, or 8.0.

The kinetic parameters for hydrogen production from a mixture of acetate and butyrate under different pH levels are shown in Table 6-2. Increasing the initial pH resulted in reduced lag time (λ) and improved hydrogen production. The average λ was 120 h at pH 4.9, which was reduced to 31 h at the initial pH 8.0 (Table 2).

Table 6-2. Kinetic parameters for hydrogen production from fatty acids mainly composed with acetate and butyrate at an initial solution pH of 4.9, 6.8, or 8.0

Initial pH	Final pH	λ[a] (h)	R_m[b] (mL/h)	P[c] (mL)	Specific H_2 production rate (mL/(g-VSS d)
4.9	5.2	120	0.07	1.4	2.3
6.8	7.2	57	0.24	26.6	15.65
8.0	8.4	31	0.49	45.2	23.79

[a] lag time

[b] maximum hydrogen production rate

[c] hydrogen production

Significantly longer lag phase combined with the poor cell growth at pH 4.9 indicates that microalgal metabolism is in favor of neural or alkaline solution pH for *M. reisseri* YSW05. These results suggest that the initial pH should be adjusted to neutral to slightly alkaline condition for improved microalgal growth and subsequent hydrogen production. Table 6-2 also shows the pH dependence of specific hydrogen production rate demonstrating that the productivity was ca. 10

times higher with the solution pH 8.0 than H_2 production achieved for the cultivation of microalgae at pH 4.9.

The maximum conversion of the fatty acids by *M. reisseri* YSW05 to hydrogen was achieved at pH 8.0, accounted for 28.2% of the total consumed acetate. This conversion efficiency was comparable with the results (51.6%) from a previous study using *Rhodopseudomonas sp*. with commercial grade acetate as a carbon source (Chen et al., 2008) while much smaller conversion efficiency was also reported by *R. sphaeroides* (7.6%) (Barbosa et al., 2001). The activity of microalgal H_2ase can be assessed by measuring the hydrogen dependent reduction of the artificial electron acceptor MV (Yu et al., 1969). A review of the literature reveals that an important feature of the hydrogen enzyme system for wild type bacteria is the capability to channel all available electrons towards hydrogen production in the absence of NADH dehydrogenase (Dutta et al., 2005). Trchounian et al. (2011) also reported that no significant difference was found in the growth of wild type bacteria or the H_2ase gene mutants under different pH between 5 and 9 where its growth rate and pattern were similar. The specific activity of microalgal H_2ase was improved with increasing the solution pH from 4.9 to 8.0, which resulted in enhanced photo-heterotrophic hydrogen production. The highest H_2ase specific activity (22.2 U/mg protein) was observed for the cultivation of *M. reisseri* YSW05 at pH 8.0 (Table 6-3) coincided with a significant cell growth (increased from 0.6 to 1.9 g/L as appeared in Fig 2 B).

Table 6-3. Specific activity of H_2ase during the photo-heterotrophic production of H_2 by *M. reisseri* YSW05

Anaerobic wastewater effluent	Total activity (Units)	Total protein (mg)	Specific enzyme activity (U/mg of protein)
pH 4.9	68.63	14.25	4.82
pH 6.8	208.99	16.24	12.87
pH 8.0	395.11	17.72	22.23

The poor H_2ase activity along with significantly low H_2 production observed with pH 4.9 might be due to the slower metabolic activities resulted in very poor cell growth and mass production. This indicates that although the activity of H_2ase is not directly influenced by solution pH, H_2 production can be indirectly influenced by poor cell growth and mass production.

6-3-3. Hydrogen production by microalgae from renewable wastes

The ability to reuse organic-enriched wastewaters that are abundantly available from municipal and industrial treatment facilities is one of the greatest advantages applying microalgae to hydrogen production, making photo-heterotrophic condition more attractive among the cultivation strategies for improving the microalgal growth. It would be obviously cost-prohibitive to use commercial organic compounds (e.g., glucose) as a primary substrate to stimulate heterotrophic bacterial hydrogen production (Min and Sherman, 2010; Yeager et al., 2011). Our results demonstrate microalgae can elicit a hydrogen production from organic-enriched waste streams and thus reducing operating expenses for microalgae cultivation and impact on the environment by producing a smaller volume of wastes.

6-4. Conclusions

Fatty acids enriched fermentor effluent mainly composed with acetate and butyrate were collected from a conventional anaerobic fermentor at a local wastewater treatment plant, and subsequently treated and used for microalgal photo-heterotropic H_2 production. *M. reisseri* YSW05 showed the potential for H_2 production using an acetate/butyrate mixture, and highest H_2 production was observed at pH 8.0 where both cell mass production and the specific activity of H_2ase were greater than the values with other culturing pH conditions. Increasing the initial solution pH from 4.9 to 8.0 resulted in improved enzyme

activity and a consequent boost in hydrogen production during the photo-heterotrophic growth of *M. reisseri* YSW05. A substantial uptake of the fatty acids by the microalga occurred at an initial pH 6.8 or higher, which was consistent with the highest hydrogen production (31 μmol/mg Chl *a*/h) accomplished at pH 8.0. These results demonstrate that the fatty acids can be used as a valuable feedstock for aerobic photo-heterotrophic cultivation of microalgae and subsequent bioenergy production. The economic evaluation of using the renewable carbon sources for promoting algae growth with concurrent bioenergy production should be further investigated.

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CHAPTER 7

Conclusions

The feasibility of hydrogen production from microalgae and fermenting bacteria was demonstrated with respect to direct hydrogen generation under aerobic conditions and the reuse of organic-enriched waste water by microalgae, and improvement of hydrogen production using enriched sulfate waste and ideal two-stage (H_2/H_2) process. The ability to organic-enriched wastewaters that are abundantly available from municipal industrial treatment and fermenting effluent facilities is one of the greatest advantages applying fermenting bacteria and microalgae to hydrogen production, making dark-fermentation and photo auto-/hetero-trophic conditions more attractive among the cultivation strategies for improving the microorganism growth. Our results demonstrate microalgae can elicit a hydrogen production from organic-enriched waste streams and thus reducing operating expenses for microorganism (i.e., fermenting bacteria and microalgae) cultivation and impact on the environment by producing a smaller volume of wastes. It will be beneficial to further improvement of hydrogen production in large-scale applications; especially microalgae produced hydrogen by utilizing H_2 fermentative effluents or various wastewaters as the substrate, which seems to be more advantageous for economic cost. The work provides an evidence of naturally evolved oxygen-tolerant hydrogenase in eukaryotic microalgae, and further study may open a new avenue of continuous biophotolysis for hydrogen production and an opportunity to apply artificially designed oxygen

tolerant hydrogenase-based biomimetic photovoltaic cell using eukaryotic algae. The ability to reuse organic-enriched wastewaters that are abundantly available from municipal and industrial treatment facilities is one of the greatest advantages of using microalgae for hydrogen production, making photo-heterotrophic condition more attractive among different cultivation strategies used for improving the microalgal growth. Fermentative hydrogen studies showed that the specific sulfate reducing activities significantly influenced the biological H_2 production and a highly efficient hydrogen recovery was obtained in a two-stage fermentation system. The conclusions of this research can be summarized as follows:

7-1. Hydrogen Production from Sulfate- and Ferrous-Enriched Wastewater

a) The presence of SRB had little influence on hydrogen production (1.7-1.9 mol H_2/mol glucose) at a specific sulfate reducing activity (0.1 g TS/g SRB h) or less with variations in pH (5.8-6.2).

b) Biohydrogen can generate successfully (1.6-1.7 mol H_2/mol glucose) even wastewater have high concentrations of sulfate by adding ferrous ions and under the controlled pH (pH 5.8-6.2).

7-2. Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system

a) The fermentative H_2 production from rotten fruit wastewater amended with digested sewage sludge showed a promising H_2 production.

b) The sequential two-stage H_2 fermentation process was demonstrated to be very efficient to recover up to 97% of the calculated H_2 production for apple and pear rotten wastewater amended with digested sewage sludge.

c) The experimental results on fermentative H_2 production were well explained with Gompertz equation (i.e., r^2>0.94)

7-3. Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions

a) The photoautotrophic hydrogen production was greatly enhanced when supplemented with high concentrations of CO_2.

b) Fe-hydrogenase in these microalgae was possibly tolerant to high levels of oxygen and thus the activity of hydrogenase was not completely inhibited even when atmospheric levels of oxygen existed.

c) Relative expression of mRNA synthesis of *hydA* and the specific activity of hydrogenase revealed that hydrogen production was facilitated by hydrogenase expression even when an atmospheric level of oxygen existed.

c) Photoautotrophic hydrogen production by eukaryotic algae under different oxygen levels (including atmospheric condition) is ecologically important where the massive growth of algae can change the local environmental conditions in terms of H_2 bioavailability.

7-4. Photo-heterotrophic hydrogen production by a microalga from acetate- and butyrate- enriched wastewater

a) *M. reisseri* YSW05 showed the potential for H_2 production using an acetate/butyrate mixture, and highest H_2 production was observed at pH 8.0.

b) Increasing the initial solution pH from 4.9 to 8.0 resulted in improved enzyme activity and a consequent boost in hydrogen production during the photo-heterotrophic growth of *M. reisseri* YSW05.

c) A substantial uptake of the fatty acids by the microalga occurred at an initial pH 6.8 or higher, which was consistent with the highest hydrogen production (31 μmol/mg Chl a/h) accomplished at pH 8.0.

국문요약

광합성반응과 혐기발효를 이용한 바이오 수소 생산에 대한 이해를 높이기 위해서는 미생물 물질대사, 무/유기광합성, 대체에너지와 유기물처리 기술 및 생화학 등의 다양한 연구가 필수적이다. 생물학적 수소 생산은 미세조류와 광학미생물을 이용 광합성반응과 광합성 미생물에 의한 유기물 분해법, 유기물질로부터 혐기발효 수소생산과 광합성 미생물과 혐기성 미생물을 조합한 조합공정이 있다. 특히, 생물학적 수소 생산으로 효율이 좋은 암반응 수소 발효는 점차 수요가 증가하고 있다. 그러나 대량 수소 생산을 위한 규모 증가에서 혐기발효 공정은 경제성 때문에 실패되었다. 이와 달리 2 단 공정은 생물학적 수소 생산을 증가 시키는 하나의 해결방안으로 암반응 수소 발효의 효율성을 증가시켰다. 하지만 혐기발효공정은 온실가스인 이산화탄소가 동시에 생산되어 이산화탄소 처리 기술이 필요하다. 이산화탄소 제거는 국제적인 이슈로써, 본 연구의 다른 목적으로 이산화탄소를 조류에 적용하여 바이오 에너지생산을 목포로 하고 있다. 바이오 수소 생산의 다른 방법인 광합성 반응은 미세조류를 이용한 수소 생산은 이산화탄소를 이용한 광학 유기 또는 무/유기를 사용하는 것을 중점적으로 산소가 제한된 혐기 조건에서 진행되어 왔다. 또한 혐기성 유출수에 함유된 영양염분 (지방산)을 에너지원으로 사용하여 미세조류를 배양과 수소생산을 목적에 두고 있다. 다시 말해 호기 조건에서 미세조류 배양 동안 광합성을 이용하여 직접적 수소 생산을 검토하고, 혐기발효공정 유출수에 함유된 아세테이트와

뷰티레이트를 이용한 광합성 유기 반응의 이해와 동시에 최적 유기성 기질 소모에 대한 미세조류의 수소 생산에 대한 이해를 중점적으로 검토하였다.

본 연구의 최종적 목표는 유기폐수를 사용하여 최대 에너지를 회수하는 신개념 기술 개발을 목적으로 유기폐수로부터 에너지 회수를 위한 과학적이며 기술의 통합적인 전체 공정을 모사하고자 한다. 따라서 다양한 유기성 폐수 (황산염 함유, 과일폐수)에서 수소 생산을 위한 최적 혐기 발효 조건 파악 및 무기/유기 탄소원을 이용한 미세조류로부터 직접적인 수소 생산을 검토하였다.

더욱 자세하게는 연속 수소 발효에서 pH 와 다양한 철, 황산염 농도 변화에 따른 황산염 환원균과 수소 생산에 대한 연관성을 검토하였다. 유기성 폐수 내에 철과 황산염은 수소 생산 효율을 증가시켰으며, 적절한 황산염과 철 성분이 함유된 폐수를 이용 시 수소 생산효율의 증가를 의미한다. 또한 반응기 내에 기질 소모에 따른 황산염 환원균의 정량화를 검토하여 수소 생산에 가장 이로운 조건을 모색하였다. 황산염 환원균은 pH 6.2 에서 수소 생산을 억제하였으며, pH 가 5.5 로 저감됨에 따라 황산염 환원균도 감소하여 황산염 환원균의 활성이 발생되지 않았다.

이단 발효 공정 시스템은 수소 생산에 대한 에너지 효율을 증가 시킬 수 있으며 과일 폐수로 연구해 본 결과 일단인 4.6%에서 이단인 경우 15.5%로 에너지 생산이 증가하였다.

미세조류에서 직접 수소 생산에 대한 연구 결과 수소화효소 (*hydA*)의 합성에 의한 mRNA 는 수소 생산과 직접적 영향이 있으며 호기성 조건에서

연속 빛 조사 시 이산화탄소를 탄소원으로 사용하여 광합반응을 통해 수소 생산이 가능한 것을 보였다. 본 연구에서는 자연 상태에서 수중에 용존된 산소 농도를 적용하였으며, 수소화효소는 대기조건에서도 효소반응을 하여 수소 생산의 가능성을 입증하였다.

혐기성 반응기 유출수에 함유된 아세테이트와 뷰틸산을 미세조류 성장 기질로 사용하여 수소 생산 가능성을 조사한 결과 아세테이트와 뷰틸산은 미세조류가 사용하기 좋은 기질로 나타났다. 수소 생산 결과 혐기성 유출수를 사용하지 않은 대조군에 비해 약 1.8 배 높은 수소를 생산하였다. 본 연구 결과 미세조류 배양과 수소 생산에 혐기성 유출수는 좋은 기질로 판단된다.

Key words: 수소; 광합성반응; 혐기발효; 미세조류; 혐기성 균주; 독립영양체; 수소화효소; mRNA 반응; 황산염환원균; 이단 공정